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# CALCIUM

## The Grand-Master Cell Signaler

proliferation

differentiation

DEATH

James F. Whitfield
Balu Chakravarthy

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# Calcium:
# The Grand-Master Cell Signaler

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# Calcium:
# The Grand-Master Cell Signaler

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**and**
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*National Research Council of Canada*
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# Contents

Abstract/Résumé . . . . . vi

Introduction . . . . . 1

1. Cell Cycles and Checkpoints. . . . . 11

2. Calcium — A Cell Cycle Driver . . . . . 43

3. Calcium and Keratinocyte Diffpoptosis . . . . . 75

4. Calcium and Colon Cell Diffpoptosis . . . . . 141

5. Calcium and Neurons . . . . . 177

6. But What About $Ca^{2+}$ and Plants? . . . . . 229

7. Summary . . . . . 237

Index . . . . . 239

# Abstract

This unique book has an exciting story to tell about $Ca^{2+}$, the versatile Master Cell Signaler whose influence reaches into all corners of the cell and controls cell proliferation, differentiation, cell functions too numerous to list here, and even cell death. The book begins nearly 4 billion years ago with the emergence of life on the newborn Earth. One of the great turning points in the history of life on Earth, *The Great $Ca^{2+}$-Driven Eukaryotic Revolution*, happened about 2 billion years later when $Ca^{2+}$ was converted from a dangerous killer that had to be kept out of cells into the versatile Grand Master of cell signalers. It was this revolutionary conversion that enabled the development of eukaryotic cells from which came animals with shells, internal and external skeletons, muscles and brains, and, of course, the plants.

The reader will be taken on a tour of tissues chosen to best show three (proliferator, differentiator, killer) of $Ca^{2+}$'s many faces. The reader will first see $Ca^{2+}$ and its many helpers, such as the $Ca^{2+}$-binding signaler protein calmodulin, directing the key events of the cellular growth-division cycle. Then the tour will move onto the skin and then the colon to show $Ca^{2+}$ driving the proliferation of progenitor cells, then the differentiation and ultimately the programmed death of their progeny. Also, the reader will learn of the striking disabling and bypassing of $Ca^{2+}$-dependent control mechanisms during carcinogenesis. After this, the reader will travel up to $Ca^{2+}$'s favorite playground, the brain, to watch the ion drive the proliferation of neuroblasts to build the basic brain, steer axonal growth cones to their targets, trigger the release of neuotransmitters, provide the structural basis of memory by helping reconfigure plastic synaptic junctions, and excessively accumulate in, and kill, overexcited neurons in the stroked brain. Finally, and perhaps unexpectedly for such a book, the reader will be shown how $Ca^{2+}$ and its helpers drive the proliferation of plant cells, trigger the responses of plants to touch, wind, and drought, and steer the pollen tube to its ovarian target, just as they steer the axonal growth cone to its target neuron.

# Résumé

Dans ce livre unique en son genre, on fait le fascinant portrait du $Ca^{2+}$, transmetteur cellulaire fondamental de vaste portée qui joue un rôle dans tous les aspects de la vie cellulaire, notamment dans le contrôle de la prolifération, de la différenciation et même de la mort cellulaire. Le récit commence il y a quelque 4 milliards d'année, à l'aube de l'émergence de la vie sur notre planète juvénile. Un des grands tournants de l'histoire de la vie sur Terre, soit *la grande révolution des eucaryotes mue par le* $Ca^{2+}$, a eu lieu 2 milliards d'années plus tard, lorsque le $Ca^{2+}$ est passé de l'état de poison à celui d'élément clé de la transmission intracellulaire. C'est cette conversion extraordinaire qui a marqué l'organisation des cellules eucaryotes en organismes munis d'une coquille, d'un squelette interne ou externe, de muscles et d'un cerveau ainsi que, bien sûr, en organismes végétaux.

Le lecteur a droit, dans cet ouvrage, à un examen de tissus rendant le mieux compte de trois des multiples facettes du $Ca^{2+}$, soit ses facettes proliféatrices, différenciatrices et destructrices. Il sera ainsi témoin de l'orchestration, par le $Ca^{2+}$ et par ses assistants, y compris la calmoduline, protéine de transmission de liaison avec le $Ca^{2+}$, des principales étapes du cycle de croissance-division cellulaire. L'initiation se poursuivra dans les cellules de la peau et du colon, où sera mis en lumière le rôle du $Ca^{2+}$ dans la prolifération des cellules progénitrices, dans la différenciation des cellules descendantes puis dans la mort programmée de ces dernières. De plus, le lecteur se familiarisera avec l'inhibition et le contournement des mécanismes de contrôle dépendant du $Ca^{2+}$ dans le contexte de la carcinogénèse. Ensuite, le lecteur sera transporté au parc d'attraction du $Ca^{2+}$, c.-à-d. le cerveau, et constatera comment l'ion contribue à la prolifération des neuroblastes afin de constituer le cerveau, d'orienter les cônes de croissance axonale vers leur cible, de déclencher la libération de neurotransmetteurs, de fournir les fondations structurales de la mémoire en aidant à reconfigurer les synapses plastiques et d'inonder et de tuer les neurones surexcités du cerveau apoplexique. Enfin, ce qui en surprendra peut-être plus d'un, on traite dans l'ouvrage de la façon dont le $Ca^{2+}$ et ses assistants dirigent la prolifération des cellules végétales, déclenchent la réaction des plantes au toucher, au vent et à la sécheresse et orientent le tube pollinique à sa cible ovarienne, de la même façon qu'ils orientent le cône de croissance axonale vers son neurone cible.

# Introduction

*"Ja, Kalzium das ist alles" ("Yes, Calcium, that's everything")*

— O. Loewi[1]

*"A small ion with cachet; . . . that old signal crackerjack"*

— W. Loewenstein[1]

There have been many revolutions, but none have had as great an impact on this planet and ourselves as the *Great Calcium-Driven Eukaryotic Revolution* (GCDER) that happened around 3–1.8 Ga (i.e., $3 \times 10^9 - 1.8 \times 10^9$ years) ago.[2,3] The events leading up to that momentous revolution started fast and furiously after the birth of the planet, about 4.5 Ga ago, perhaps on the surfaces of the plentiful clay crystals or iron pyrites which collected, concentrated, and catalyzed the interaction of the many inorganic and organic molecules being forged in the hot young planet or were coming preformed in the deluge of accretion rocks that were building the planet. In time, relatively impermeable, proto-lipid membranes came to enclose such crystal-bound concentrates within which networks of reactions for making, maintaining, and replicating the new living things could then be established and retained.[4–7]

There was no lack of energy in the neonatal planet to drive these reactions. There was solar energy, but this was a mixed blessing because "sun-blockers," such as a layer of water and mucilages, were needed to reduce the intensity of the sunlight and protect the nascent structures from prompt demolition, since there was no ozone layer in the as yet oxygen-less world to shield them from UV. Until 4 Ga ago, there were massive showers of space rocks, the impacts of which periodically melted large parts of the planet and converted the oceans to steam and destroyed any protocells. In between the bombardments, there was much $CO_2$ from the "out-gassing" volcanoes that were making and pumping out the planet's new atmosphere (the planet was much too small to retain the hydrogen that was the largest part of its natal gas cloud), and since nothing had yet been invented to use it, it caused a greenhouse warming. There also were volcano-generated pyrophosphates, the forerunners of ATP that made their way into the crystal-bound concentrates.

When the spatial bombardments finally subsided 3.8 Ga ago, biogenesis could start yet again, but at last it could proceed without interruption. However, the spatial bombardments had done one positive thing. They had delivered a lot of water and organic molecules, including some L-amino acids, which had been produced in the cold (−400°C) solar system-generating interstellar cloud from CO, $CO_2$, methanol, and hydrocarbons that had been confined in ice-encrusted silicate granules and pushed by stellar radiation to react with each other.[8] These extraterrestrial organic molecules may

have kick-started and initially supplemented the soon to become massive generation of terrestrial biomolecules. The progenitors of future biomolecules were made by cycles of molecular breakdowns and polymerizing condensations during heating and drying on the sides of volcanoes but perhaps more importantly in and around deep seafloor vents (e.g., the hot "smokers") where superheated water under immense pressure (which prevented destructive boiling) flowing over the crust created (and still do create) new molecules, clays, and even membrane-bound structures. Some of the creations of these ovens collected on the ionically patterned surfaces of micro-chip-like catalytic clay crystal platforms to produce, after countless attempts and failures, the first successfully *metabolizing* protocells, which could crudely divide when they had reached a certain size. At some point after 4 and before 3.7 Ga ago, at least one of these metabolizing "cells" was "infected" with one of the products of the seafloor ovens — a very primitive and very error-prone device that at first could only replicate itself but eventually became able to assemble specific chains of amino acids for the metabolic peptides.

This device was the forerunner of RNA, possibly a PNA (peptide nucleic acid), consisting of a chain of nucleic acid bases linked to a backbone of the then prevalent polymeric *N*-(2-aminoethyl)glycine instead of the later-developed chain of ribose molecules.[9] Already at this time the first trinucleotide PNA had appeared, which could select among the few available prebiotic amino acids.[9] This PNA-based device and its RNA successor were "ribozymes," which could cut and paste themselves, drive their own replication, and at the same time be a two-dimensional memory tape, which could translate the information coded in nucleotide sequences into structural and functional reality by reconfiguring into a three-dimensional platform for actively selecting and linking amino acids into protein chains.[1,10] However, things were moving so incredibly "fast" that by 3.5 Ga ago (when the planet was "just" 1 Ga old) the waters were swarming with "modern" cells (including the oxygen-generating photosynthetic cyanobacteria), the cooked coaly remnants of which have been found in the Apex cherts from the Precambrian Pilbara supergroup in Northwestern Australia.[7,11] Sometime between 4 and 3.5 Ga, after the massive spatial bombardments had stopped, the ancestors of these Apex chert cells had also figured out how to park their growing load of information in an inert two-dimensional, highly condensable, double helical tape, DNA, from which the various bits of the information could be retrieved and transcribed back into the now "old" RNA from which they could then be translated into the various enzymes and other proteins when needed.[1,10] But how did the cells learn to replicate these double-stranded DNA information tapes? This was not easy! It must have required a lot of trial and error to become efficient. They had to invent a pack of proteins that collect into a replication factory attached to the cell membrane that recognizes unwinding sites (replication origins), unwinds the double helix, holds the unwound strands apart, and then achieves the remarkable gymnastic feat of replicating the differently oriented strands in opposite directions at the same time, using disposable RNA primers and different polymerases and ligases for different aspects of the job and editing out errors in the new strands as they are being made. Therefore, these Ga-old cyanobacteria were already very sophisticated and were using what had become the universal standard polyphosphate-powered metabolic system. They started two very important things: they were the ones that started pumping streams of oxygen into the atmosphere as a byproduct of their photosynthetic hydrolysis of water to get protons for the steep transmembrane proton gradients needed to make their ATP biofuel,

and they put the brakes on any potential runaway greenhouse heating (as was happening at the same time on Venus) by using the volcano-produced $CO_2$ to make glucose and body parts and locking it up in calcium salts.

Indeed, there was a lot of $Ca^{2+}$ (3–10 mM) in those ancient seas. At first, it was a dire menace.[3,12] It was a formidable obstacle to the protocells developing the now familiar, universally standard $Mg^{2+}$- and phosphate-based metabolic systems.[1,3,11] All of the modern metabolic systems work by accumulating an organic polyphosphate, ATP, to produce a high ATP/ADP ratio that is so very far from equilibrium that coupling a reactant to ATP can displace the overall reaction equilibrium by as much as $10^8$-fold in favor of the product(s), i.e., drive the reaction forward to de facto irreversibility. However, before they could do this and before the cyanobacteria could be invented, the protocells had to do something about the huge amount of $Ca^{2+}$ in the seawater, direct exposure to which, unlike an exposure to $Mg^{2+}$, would instantly nip any such reactions in the bud by forming insoluble complexes with the phosphates.[2,7] This $Ca^{2+}$ would also impede membrane formation by causing fatty acids to collect into useless soapy "globs."[7] Therefore, they had to find ways to keep as little as possible of the ion inside themselves. They had to reduce its level to around $10^{-5}$–$10^{-7}$ M. How they did this without the sophisticated high-tech pumps, channels, and antiporters of today, or even those of 3.5 Ga ago, is unknown. However, dangerous though $Ca^{2+}$ was (and still is, as any crippled stroke victim with masses of $Ca^{2+}$-killed neurons can tell you), it was unlike all other ions in having a hidden gift for triggering a vast variety of functions if only someone could figure out how use it safely. This gift stems from its remarkable flexibility.[12] Because it can have as many as 10 and as few as 6 coordination numbers, it can cross-link a wide range of inorganic and organic crystal structures.[1,3,12] This unique flexibility enables it to be a master signaler and driver of cellular programs, which can easily slip out of its watery shell and climb into the regulatory pockets of many target proteins to reconfigure and activate them.[1,3,12]

The anaerobic pre-prokaryotes were able to make organic anions in water (using energy stored in pyrophosphates) inside a membranous container, which had to be free from $Ca^{2+}$.[12] However, $Ca^{2+}$ was an excellent tool for stabilizing and driving devices on the cell surface. The extremely successful cyanobacteria, the Lords of Precambrian Life and Waters, which are still with us,[11] were already using $Ca^{2+}$ 3.5 Ga ago to do important outdoor jobs. For example, they were using a surface protein (Swm A, oscillin) with multiple $Ca^{2+}$-binding sites to drive their gliding and swimming.[13,14] The ancestors of some modern bacteria have even learned to use $Ca^{2+}$ for some indoor jobs. Thus, *Escherichia coli* lowers or raises its internal $Ca^{2+}$ concentration to respectively steer itself toward chemoattractants or away from repellants.[15,16] (Interestingly, our central neurons still bear the traces of this ancient $Ca^{2+}$-dependent food-seeking guidance system in their receptors that are activated by amino acids such as glutamate, glycine, and aspartate, and that we will talk about in Chap. 5.) They also use $Ca^{2+}$ fluxes to trigger DNA replication, segregation of the nucleoids, and cell division.[17]

The pre-Apex chert, pre-cyanobacterial Last Common Ancestor (LCA), or far more more likely the gene-swapping, gene-collecting, and genome-mixing cells of the CACPC (Common Ancestral Community of Primitive Cells) ancestors[18,19] of the bacteria, archea, and eukaryotes (around 3.7 Ga ago[7]), probably chemotropic and anaerobic (and maybe thermophilic) prokaryotes, were equipped with the DNA–RNA memory-recording/memory-retrieving system and a sufficiently elaborate molecular machinery

to replicate it. They also had developed chemiosmotic devices that pumped out $H^+$ to make a steep gradient of protons, which flowing down "ATPase penstocks" in the membrane generated ATP biofuel (or its pyrophosphate predecessor), drove the uptake of essentials from the outside, and ejected the constantly inwardly pushing $Ca^{2+}$ to prevent it from reaching lethal levels and yet use it to do important things.[20] However, this accumulation of various ions and small molecules presented the emerging cells with a serious problem — osmotic swelling and rupture. The cells had to find a way to avoid being destroyed by osmotic swelling. They actually devised two pressure-managing strategies. The strategy chosen by those that founded the bacteria and archaea was simply to wrap themselves in a proteoglycan "chain-link corset" or "exoskeleton."[20] But others, our founders, developed something different — an endo-fibrillar or cytoskeletal network, the EFN. It was this EFN that triggered the GCDER.

While their rigid corsets kept the bacteria and the other prokaryotes small, unicellular, and forever feeding chemiosmotically on thin soups of ions and small organic molecules, a mechanosensitive, strain-responsive EFN of contractile fibrillar proteins attached to the cell membrane allowed the novel others, the Archeons, to fight osmotic swelling by restraining membrane expansion. The genes for this EFN appear not to have come from bacteria or archaea — they seem to have come "out of the blue."[18] However, they could have come when an ancestral Archeon swapped genes with a member of a now vanished group.[18,19] This EFN paved the way for the development of elaborate interconnecting, intercommunicating compartments such as the nucleus, endoplasmic reticulum, and various vesicles and vacuoles for efficiently carrying out an ever-growing portfolio of functions. The EFN enabled them to get much bigger and provided the machinery for crawling around in search of a wide variety of foods, ranging from the old soup of ions and small molecules to more solid fare such as whole bacteria and fellow eukaryotes.[12,20] One of the results of this ability to eat bacteria and other whole organisms was the addition of the food's genes to the eater's gene pool to produce chimeric genomes.[19] $Ca^{2+}$ was poised to get inside the emerging cells and seize control of their elaborate sets of fibrils, compartments, and motors that move them, feed them, replicate their chromosomes, and ultimately divide them.[3,12] $Ca^{2+}$ then made its move and thrust its controlling tentacles into every corner of the cell and along with this even became one of the players in the regulation of the planet's climate.

Eventually, clusters of choanoflagellates evolved into the first metazoans, which invented the body-planning homeobox genes and gave rise to flatworms, arthropods, and most importantly for us, our protochordate ancestor, the amphioxus-like *Pikaia*.[21] Of course, calcium was a key player in the great changes that followed. One of the most stunning events in the history of life — the so-called "Cambrian Explosion" — was the emergence about 550 Ma ago of animals with hard, calcium carbonate- or calcium phosphate-mineralized shells and exoskeletons, for bracing the muscles that drive antennae, gill supports, and limbs, to shelter internal organs, and of course, to provide defense against predators.[21] Then came some of calcium's greatest triumphs — endoskeletons of bone and teeth. The growing masses of organisms in the oceans with calcium carbonate shells and calcite crystal armor plates eventually became large enough to affect the plant's climate by locking up in their shells the $CO_2$ in the bicarbonate from the chemical breakdown of silicate rocks washed by rain into the oceans. Thus, for example, when the temperature dropped and the planet grew glaciers, as it did 600 million years ago to

become a giant snowball, the populations of $CO_2$-sequestering shell makers shrank and the flow of bicarbonate into the ice-covered oceans stopped.[22] However, volcanoes continued to pump out $CO_2$, which accumulated in the atmosphere rather than being locked up in shells.[22] The buildup of this "greenhouse" gas eventually reached a level that caused the Precambrian planetary snowball to melt and then overheat.[22] But the increased evaporation of the hot oceans and the resulting increased rainfall increased the flow of bicarbonate into the oceans. Thus, the great heat was ultimately reduced by the regrowth of the shell makers that locked up the $CO_2$. Since then, $Ca^{2+}$ and the shell makers have been active participants in the planet's $CO_2$-buffering system.

The steep inwardly directed $Ca^{2+}$ gradient inherited from the CACPC is a kind of high-voltage storage battery which provided a steady basal $Ca^{2+}$ current with which the emerging, more elaborately organized, and highly mobile eukaryotes could operate the cellular integrated circuit that maintains and coordinates compartmental activities and which they could tap to produce different patterns of $Ca^{2+}$ puffs, sparks, and waves to trigger various cellular functions in response to external events: $Ca^{2+}$ connected the eukaryotic cells to the outside world whatever and wherever that would be — it became the cells' ionic eyes and ears.[3,12] Thus, $Ca^{2+}$ ions, squirting at high pressure (voltage) into the cell through selective channels with controllable gates in the mobile cell membrane and out of internal storage vesicles and vacuoles, triggered the endoskeleton-driven contortions of the cell surface required for crawling, drinking (pinocytosis), and feeding (phagocytosis). However, these contortions in the early "pre-eukaryotes" also produced mutants because they strained and damaged the chromosome, which was still tethered along with the replication mechanism to the cell membrane just as it was in the immobile membranes of the ancestral prokaryotes.[23] Although it had the positive advantage of accelerating evolution, this mutation-enhancing anchorage of the nucleoid to a bucking surface (like a cowboy riding a bucking bronco) was far more often dangerously destabilizing. But the chromosome was probably often inadvertently dragged into the relatively quiet and much safer cellular interior on a phagocytosed patch of the cell membrane. Then, at some point a cell(s) finally took advantage of the stability offered to its chromosome by enclosure in its own membranous sac, which was tethered to the endoskeleton to keep it in place. Thus, a new organelle was born — the true, or eukaryotic, nucleus — which opened the way to a vast increase in the chromosome (DNA) complement and many more genes for many more things.[23]

According to another scenario[23a] based on "homology-hit" genome analyses, the eukaryotic nucleus was born when an archaeal cell invaded a bacterium and settled down into a permanent parasitic existence where it depended on the bacterial metabolic system but not the bacterial genes for DNA replication and translation. In subsequent generations the archaeal parasite eventually lost its own unused metabolic genes while the chimerical cell came to depend on the archaeal gene products for DNA replication and gene expression. The upshot of these events was the appearance of eukaryotic cells such as modern yeast cells whose genes for nucleus-related functions such as replication, transcription, cell cycle, nuclear architecture, and ribosome generation still bear the evidence of their archaeal origin while the genes for cytoplasmic functions such as metabolism, stress responses, and protein and ion transport bear the traces of their bacterial origin.[23b]

According to the currently popular biosymbiogenesis hypothesis invented by Merezhkovsky[24] in 1909 and greatly embellished by Margulis and others[18,19,25–27], at some

point before, after, or during the appearance of the true nucleus, one of the new $Ca^{2+}$-driven "Archeon" cells ate, without digesting, or became infected with, one of the oxygen-generating photosynthetic cyanobacteria that settled down in its new home and became the progenitor of the modern chloroplasts and (or) an α-proteobacterium that became the progenitor of the mitochondria which endowed the resulting cells with powerful aerobic energy-capturing and -storing capabilities, and a virtually limitless range of new activities.[2,12,18,19,25–27] One of the results of this potent partnership was the formation of the chimeric eukaryotic genome when the metabolism-related and other genes released from the inevitable breakdown of some of the prokaryotic partners or from other digested food prokaryotes (might we call these genes — phagogenes?) teamed up with or replaced the original Archeal partner's genes to result in the transfer of "mitochondrial" and chloroplast genes to the nucleus from which they could eventually replace and then control the production of various components of the organelles.[19,28] An extremely important advantage of this ancient indigestion and subsequent partnership was the protection of the cell from damage by reactive oxygen species by devices contributed by the prokaryotic partner. Oxygen was, and still is, extremely dangerous. Indeed, the oxygen that the cyanobacteria pumped into the atmosphere from their ponds and tidal pools probably slaughtered vast numbers of organisms except those that had never left the anaerobic natal niches such as the hot seafloor vents where their descendants still thrive in vast numbers. However, these oxygen makers and the bacteria that learned to use oxygen to make ATP must have developed the genes and the enzymes they encode to protect themselves from the otherwise deadly reactive oxygen species that are byproducts of oxidative phosphorylation. Ultimately, these bacterial genes found their way into their hosts' genomes from which they could provide the same protection for their hosts. Of course, $Ca^{2+}$ thrust its tentacles even into these prokaryote-organelles where it can stimulate mitochondrial dehydrogenases and thus control the cell's energy production.[3,12]

(It must be pointed out hesitantly and parenthetically that chloroplasts and mitochondria might not be the descendants of ancient infections or indigestions. The early prokaryotes probably had their respiratory and photosynthetic machineries and the associated DNA codes for making them up front in their membranes. The disruptive mobility of the new plasma membranes that forced the formation of the nucleus could also have forced the protective internalization of these machineries in new pouches or organelles, which contained the prokaryotic DNA codescripts that had to be rigidly conserved to make these machines and even now speak loudly of their prokaryotic pasts. However, genes from ingested and digested bacteria and other organisms would still have found their way into the early eukaryotic genomes.)

Because it can interfere with phosphate group-transfer reactions and stimulate destructive endonucleases, proteinases, and phospholipases, $Ca^{2+}$ was as dangerous as before, which was why the protocells had to find a way to expel the ion and in the process create what would become the invaluable steep inward $Ca^{2+}$ gradient.[2,3] However, the CASPC or one of their predecessors learned how to use $Ca^{2+}$ without killing itself, a discovery that opened the door to the vast world of $Ca^{2+}$ signaling that we know today. It used, and some its undoubtedly highly evolved modern bacterial descendants still use, high-affinity $Ca^{2+}$-binding proteins that are changed into an active configuration by small, hence safe, internal $Ca^{2+}$ surges to trigger and drive key functions such as active

transport, chemotaxis, DNA replication, and sulfate reduction.[15,17,29–35] Among these was one with a tightly $Ca^{2+}$ binding "helix–loop–helix" or "EF-hand" motif that would some day be used by the eukaryotes to spawn a huge family of $Ca^{2+}$-binding, function-activating or just simple $Ca^{2+}$-buffering proteins.[3,15,35,36] Among the first, and by now the busiest, of these second-generation $Ca^{2+}$-binding sensors/effectors, is the ancient calmodulin (the only one used by all of the eukaryotes) with its four clusters of oxygen atoms, which when they meet $Ca^{2+}$ at a safe intracellular $10^{-6}$ M can curl into loops, each with a 0.099-nm radius that exactly fits the ion's crystal radius.[3,15,35] ($Ca^{2+}$'s nearest competitor in a cell would be the inflexible $Mg^{2+}$, but this would be a loose fit because of $Mg^{2+}$'s 0.065-nm radius.) This $Ca^{2+}$ contact and binding causes calmodulin to switch into a configuration that can activate a wide range of target enzymes and other proteins in response to the harmlessly small $Ca^{2+}$ signal surge.[35] (Surprisingly, in one modern eukaryote, the budding yeast *Saccharomyces cerevisiae*, the essential calmodulin functions are carried out by apocalmodulin rather than $Ca^{2+}$-calmodulin.[37]) However, no cell dares to lose sight of $Ca^{2+}$'s sinister dark side. It must keep its membrane $Ca^{2+}$ pumps, the $Ca^{2+}$-binding/buffering proteins, and the $Ca^{2+}$-sequestering systems in good working order so that they can respond instantly to any dangerous rise in the internal $Ca^{2+}$ level.

This book is a summary of what little we know of some of the key roles of $Ca^{2+}$ in the many important products of the GCDER, particularly the eukaryotic cell cycle and the fascinating combination of functional differentiation and self-inflicted death by apoptosis that Whitfield has elsewhere called *diffpoptosis*.[26] However, even these two subjects are so immense that they cannot be covered comprehensively by two people and one small book. Thus, our goal was to paint a picture in the broadest of strokes of how things seem to be at the beginning of the new millennium and where they seem to be going. Starting with the cell cycle, we will meet the different cycle-driving receptor signals, protein kinases, their inhibitors and phosphatases and we will be obliged to show our certificates of chromosomal integrity to pass through several closely guarded cycle checkpoints to visit the various places where $Ca^{2+}$ and its assistants help build and then start the megamachines that replicate the chromosomes, and finally to watch $Ca^{2+}$ and the assistants drive the cell into and through mitosis and ultimately cell division. After leaving the cell cycle, we will visit several examples of $Ca^{2+}$-stimulated, post-proliferative diffpoptosis in skin, colon, and the roles of this wonderful ion in neuronal signaling, plasticity and memory, and death. These were chosen to show the incredible versatility $Ca^{2+}$. Some readers will not be pleased that we have not mentioned $Ca^{2+}$'s control of other cells such as endometrial cells and mammary gland cells, but the principles learned from our chosen examples will apply equally to these and other cells. Our tour will end in a most unusual place for this kind of book: we will briefly show how plants use $Ca^{2+}$ to respond to touch, wind, cold, and other challenges as just a tantalizing preview of the exciting things ahead in the now rapidly unfolding story of $Ca^{2+}$ in the plant world. However, while touring we must not lose sight of the fact that we, like other multicellular creatures, are all products of sperm-triggered $Ca^{2+}$ tsunamis that caused our eggs, blocked in second meiotic metaphase, to lift the block, enter anaphase, and produce haploid pronuclei waiting to join with the approaching male pronuclei and their centrioles.[38–42]

And now, like all good tourists, let's begin by looking at a map of our first destination — the cell cycle and its checkpoints. But first we must thank Professor U. Armato of

the University of Verona and an anonymous reviewer for reading the manuscript and suggesting various ways to make it better and Diane Candler of the NRC Research Press for being the editorial midwife who actually brought the book to birth. Last, but not least, we must thank two unsung heroines, our wives Barbara and Pratibha, for supporting this venture. Hopefully, the reader will end this tour of Calcium Land eager to revisit and linger longer in some or all of the sites we will have so briefly visited.

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# 1

# Cell Cycles and Checkpoints

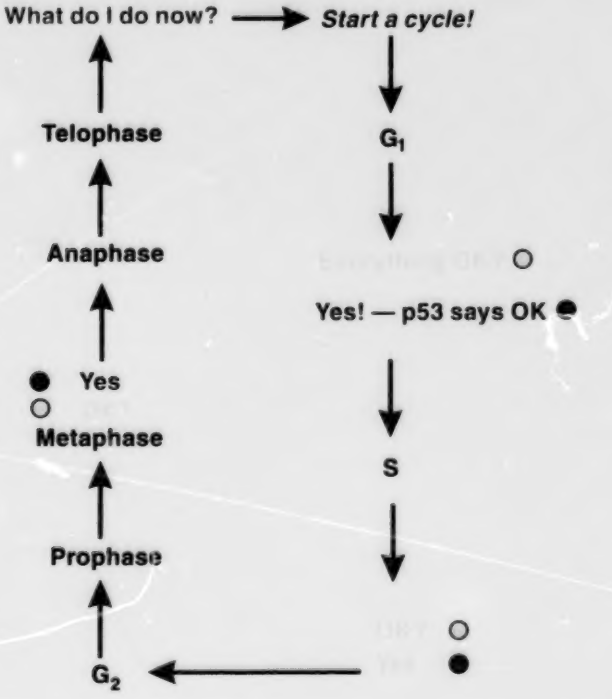
## The $G_1$ buildup: getting ready for chromosome replication

The cell cycle usually starts with a $G_1$ (i.e., first gap or "silent" period between the end of mitosis or proliferative activation and DNA replication) phase during which cresting and crashing waves of stage-specific CDKs (cyclin-dependent, serine/threonine protein kinases) drive the buildup and finally the switching on of the enormous nuclear factories that will replicate the chromosomes (Fig. 1.1).[1–15] The length of this $G_1$ buildup in a daughter cell depends on how much of the mother cell's transcriptome (the complement of various mRNA transcripts) and how many functional factory parts she has inherited. (In fact, in some parts-loaded embryonic cells there is no appreciable $G_1$ delay between the birth of a daughter cell and when it starts replicating DNA.[13]) By the end of the $G_1$ buildup, the factories are erected on the nuclear matrix, and chromosome replication origins are waiting to be grabbed and pulled into the factories where replication is switched on by the end-of-$G_1$ CDKs. The S, or chromosome replication, phase is followed by the $G_2$ (i.e., second gap or "silent" period between the end of DNA replication and mitosis) phase during which the cell makes and switches on the mitosis-specific/-triggering CDKs (Fig. 1.1). Finally, there are mitosis and cytokinesis, those few breathtaking minutes when the cell divides into two new daughter cells.

A newborn cell has five options, depending on the cues from its surroundings and its state of differentiation. It (e.g., a rapidly cycling "transient amplifying" basal skin keratinocyte or colon cell, a serum growth factor-stimulated cultured cell) may start a cycle (Fig. 1.1). It (e.g., a serum starved cultured cell) may stay in a competent-to-start-a-cycle mode, but lapse into a reversible, proliferatively comatose $G_0$ state. It (e.g., hepatocyte, thyrocyte) may differentiate and actively function, but remain able to start a cycle if signaled to do so. It (e.g., suprabasal skin keratinocytes, upper crypt colon cells) may terminally (i.e., irreversibly) differentiate, function for a while, and then commit suicide (by apoptosis); or like most (although not all) neurons, it may function efficiently for as long as 100+ years with an irremediably disabled cycle-starting mechanism as long as it does not suicidally (apoptogenically) try to activate this mechanism to replace lost neurons in a damaged or degenerating brain.

We will begin our quick tour with a reversibly quiescent ("snoozing") $G_0$ cell. Such a cell has the chromatin containing its genes for DNA-replicating components pre-configured to respond to cell cycle drivers and has pre-initiation complexes sitting on the replication origins of the first-to-be replicated "house-keeping" genes ready to be sent through the replication factories on the nuclear matrix upon the insertion of missing key components. The regulatory regions of some genes are ready for action with

**Fig. 1.1.** The cell cycle, its checkpoints. The checkpoints are shown as caution lights (○) and go lights (●). At these points, the checkpoint guardians ask questions (OK?) that demand a "Yes" answer before each transition can be allowed to proceed. Thus, for example, if G1–S guardian, p53(TP53), were to say "not OK," the buildup would stop until the damage such as a broken chromosome(s) is fixed. If the damage is irreparable, p53(TP53) would trigger the suicidal apoptogenic mechanism.




heterodimeric E2F1 or E2F3·DP isoform transactivator complexes (or other E2F-like transactivators) on "E-box" motifs (5′-T(N)T(G/C)(G/C)CGC-3′) in their promoters.[16–22] However, these genes are silent either because the C-terminus of the E2F1 or E2F3 isoform is stuck in the "pocket" of a protein of the Rb-family (the "pocket domain protein" family) and thus cannot switch on the gene-transcribing RNA polymerase II or because the E2F or E2F-like protein binds to a target gene's promoter and serves as the platform for a pocket protein that in turn holds a repressor for the gene.[14–18] Otherwise, there is an inhibitor such as Id2 that attaches to, and suppresses, the E2F·DP complexes on the E-boxes of some genes.[8,18] Contributing to the proliferative quiescence is a high level of $p27^{Kip1}$, a protein that inhibits any low-level CDK (such as the $G_1$-starting Cdk4-cyclin D1) to prevent this kinase from starting a cycle at the wrong time by inactivating pocket proteins.[11] Indeed, suppressing $p27^{Kip1}$ expression with antisense oligodeoxynucleotides prevents cycling cells from quiescing. Consequently, $p27^{Kip1}$ knock-out mice have oversized organs (organomegaly) with abnormally large numbers of smaller cells because of a shortening of the $G_1$ buildup, which prevents the cells from reaching a normal premitotic size.[8,11]

When the cell is booted into a cycle by signals from the mitogen receptors we will meet in the next chapter, it must put some new transcripts into its transcriptome to build up its ribosomal protein-making machinery and start the parade of cycle-driving CDKs. The principal agent of this buildup is the master transcription factor (TF), c-Myc. It works along with another protein, c-Max, in c-Myc·Max heterodimers that stimulate the expression of the genes coding for the following: BN51, a part of the RNA polymerase III that transcribes 5S RNA and tsRNA; eIF-4E that mobilizes and promotes the translation of growth-driving mRNA transcripts; nucleolin, a nucleolar protein involved in rRNA processing; and ornithine decarboxylase, a promoter of RNA polymerase I activity.[22a] However, the cell will also need lots of energy to grow, replicate its chromosomes and divide. To load up with glucose fuel and increase ATP energy production, the cell also uses its activated c-Myc to transactivate the genes for the glucose transporter GLUT1, phosphofructose kinase, and enolase.[22b] c-Myc·Max also stimulates the expression of the genes coding for the components of CAK (cyclin-dependent protein kinase activator kinase).[23] CAK is itself a CDK, Cdk7·cyclin H, which has at least two very important jobs: when alone, it activates the first of the cyclin-dependent protein kinases that drive the cell cycle, and at the same time, together with MAT1 (p36) it serves as the TFIIH component of the RNA polymerase II transcriber's pre-initiation complex.[2,8,23] Also booted into action is the early $G_1$-specific, CDKs-activating STY (serine/threonine/tyrosine) phosphatase Cdc25A, which, like the $G_2$-specific Cdc25C phosphatase, might be activated by a $Ca^{2+}$-stimulated protein kinase.[2,19,20,24,25] Of course, there must be a CDK for it to activate, and indeed this is provided when c-Myc·Max also turns on the genes for Cdk4 and cyclin D2, the products of which unite to make the cycle-starting Cdk4·cyclin D2.[25a,b]

At this point, we must talk about the cell's proteolytic devices and the selective proteolyses that enable the sequential cresting and crashing of the different CDKs that drive the buildup of the chromosome replication factories. There must be a period of low CDK activity at the start of the buildup, otherwise, the kinases would prevent the assembly of pre-initiation complexes (which we will meet below) by phosphorylating key components.[26] Therefore, while CKIs such as $p27^{Kip1}$ must eventually be shredded, they must not be dumped into the proteosome shredder too soon.[8] Indeed, some cells such as thyrotropin-stimulated dog thyroid cells actually *upregulate* $p27^{Kip1}$ expression, and although rat osteoblasts and IL-2-stimulated lymphoid cells do decrease $p27^{Kip1}$ expression, they upregulate another CKI, $p21^{Cip1/WAF1}$.[2,27–29] Besides giving a narrow window for the assembly of the pre-initiation complexes, and in the case of $p21^{Cip1/WAF1}$ actually promoting the assembly of Cdk4·cyclin D1 CDKs when present in low concentration, this not-so-crazy expression of a CKI early in the G1 buildup ensures that the CDKs, particularly Cdk2·cyclin E, must exceed a threshold level after which they will trigger the destruction of the inhibitor while stimulating the expression of the genes for the replication factories and triggering replication.[2,8] They may do this by phosphorylating the CKI, which marks it for polyubiquitination by the functional equivalent of the budding yeast's $SCF^{Cdc4}$ complex (which marks the yeast's S-phase-restraining Sic1 CKI for destruction) and delivery to the proteasome shredder[30], which, as we shall see later on, may have been stimulated by the cyclic AMP transient that occurs in middle to late $G_1$ phase. Of course, the expression of the inhibitors suppresses background

kinase "noise" and their subsequent destruction enables the generation of sharply defined surges of CDK activites and gives a direction to the cycle.

$p27^{Kip1}$ may also be part of a mechanism that protects the cell from the perils of trying to proliferate when it shouldn't. Thus, when the cell is low on fuel (i.e., ATP), $p27^{Kip1}$ behaves as a cycle-stopping CKI that inhibits the $G_1$ CDKs.[8] But when ATP is plentiful, $p27^{Kip1}$ becomes a substrate for phosphorylation by Cdk2·cyclin E, which marks it for ubiquitination and shredding.[8]

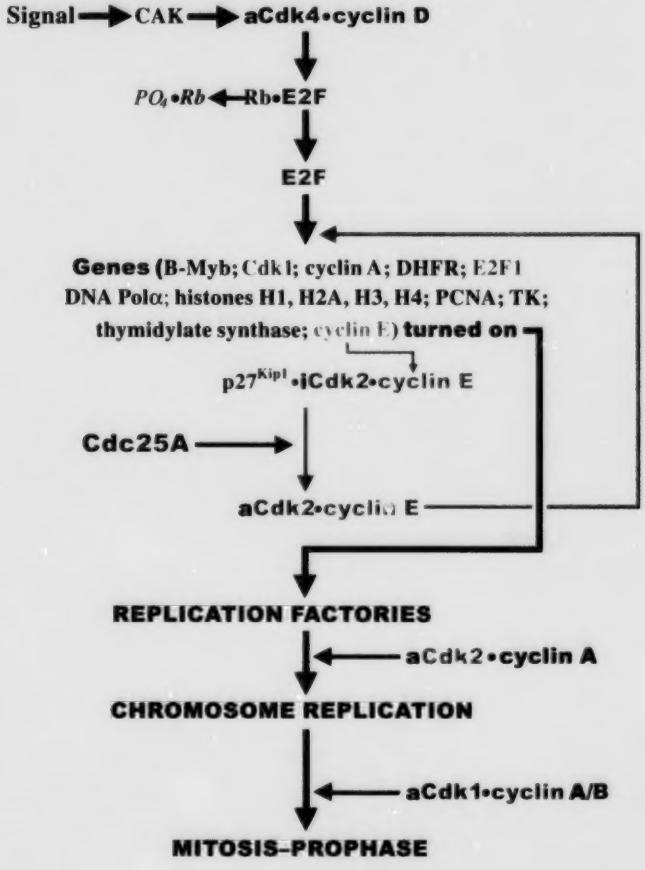
However, the ubiquitination – proteasome shredder may not be the only, or even the most important, proteolytic device used in the $G_1$ buildup in some cells. Thus, it seems that the $Ca^{2+}$-activated protease calpain is needed for the $G_1 \rightarrow S$ transition in fibroblasts and vascular smooth muscle cells.[31,32]

The first signal starts the expression and buildup of cyclin D isoforms (D1, D2, and (or) D3) (Fig. 1.2) which attach to the c-Myc-induced Cdk4 catalytic subunits to produce inactive Cdk4·cyclin D CDK holoenzymes. The new cyclins are promptly shipped from the cytoplasmic factories to the nucleus where they are localized to transcription sites on the matrix.[33] There the CDKs are activated when Cdc25A removes phosphates from threonine and tyrosine phosphates blocking the Cdk4 (or 6) catalytic domain, and CAK phosphorylates another threonine residue to shove aside a loop that was blocking the CDK's substrate-binding site. The active enzymes start building up to a peak near the $G_1 \rightarrow S$ transition (Fig. 1.2). After a delay, the E2F isoforms such as the independently controlled E2F1 and E2F3 start building up in the nucleus, to a mid to late $G_1$ peak. As they accumulate in the nucleus, they bind to DP-1, and the resulting E2F·DP-1 heterodimers bind to the promoters of many target genes such as those for Cdc6 or the pre-initiation complexes, Cdk1 (formerly known as Cdc2), cyclin E, dihydrofolate reductase, DNA polymerase-α, E2F1, histone H2A, possibly histones H1, H3, H4, B-Myb, c-Myc, DNA polymerase-δ-pulling PCNA homotrimer, RRM2 (M2 (or R2) subunit of the deoxyribonucleotide-making ribonucleotide reductase), thymidine kinase, and thymidylate synthase.[7,12,13,17a,19,20] But there they still can't stimulate transcription because they are promptly grabbed by hypophosphorylated (i.e., active) pRb and the other pocket proteins which also have been accumulating since the start of the buildup. Clearly, this is meant to prevent premature DNA replication.

The still hard-working hepatocytes in a regenerating rat liver remnant start out immediately after the partial hepatectomy with Cdk4 and cyclin D3 in their cytoplasms and cyclin D3 in their nuclei.[34] By 5 h after the surgery (which has proliferatively activated the cell by triggering the release of hepatocyte growth factor), Cdk4 and cyclin D3 have moved into the nucleus. By 13 h (midway through the prereplicative buildup), inactive Cdk4·cyclin D1 or D3 has appeared in the nucleus and started "homing" onto the chromatin to get at pocket protein targets.[34]

In some cells such as thyrotropin-stimulated dog thyroid cells, the cycle can be started by cyclic AMP and the cyclic AMP-dependent protein kinases (PKAs) without an accumulation of cyclins D1 and D2 and with an actual reduction of the basal expression of cyclin D3.[35] But wait a minute — the cell cannot replicate its chromosomes without having enough Cdk4·cyclin D to hyperphosphorylate and disable the pocket proteins!! What's going on? The cell gets around this obstacle by enhancing the transport of Cdk4 and cyclin D3 into the nucleus and increasing the affinity of cyclin D3 for Cdk4, which is

**Fig. 1.2.** The cresting and crashing waves of cycle-driving cyclin-dependent protein kinases — the CDKs. A reversibly quiescent $G_0$ cell, perhaps a serum-deprived fibroblast, is awakened to start a cycle by a signal from a mitogenic receptor, perhaps a velcroceptor such as the PDGF receptor. The signal stimulates a buildup of the first of the CDKs, Cdk4·cyclin D, the only one of the CDKs that is responsive to signals from the outside world. The signal also stimulates the expression of CAK, another multipurpose CDK that activates Cdk4·cyclin D. Thus starts the first, and the only externally triggered, wave of CDKs that phosphorylates the Rb pocket proteins blocking the expression of genes for the components of subsequent CDKs and also the components of the huge chromosome replication factories that will be assembled on the nuclear matrix during the G1 buildup. The wave of Cdk4·cyclin D1 activity subsides as Cdc25 converts i(inactive)Cdk2·cyclin E into a(active)Cdk2·cyclin E, which collaborates with E2F isoforms to turn on the genes for, and production of, the many parts of the replication factories as well as Cdk1, cyclin A, and its own cyclin E. When it has helped build the factories and completed the ground work for a mitosis it will not see, the hard-working, but short-lived, aCdk2·cyclin E wave crashes and is soon followed by one of its products, Cdk2·cyclin A, which works throughout the S phase on the replication factories' assembly lines, activating replicon origins. When the chromosomes have been replicated, Cdk2 is shredded and replaced by Cdk1, which forms the mitotic CDKs, Cdk1·cyclin A or B, that trigger mitotic prophase. The last of the CDKs, Cdk1·cyclin B, is destroyed when the signal to start anaphase is given.



functionally the same as making more CDK: the nucleus ends up with enough active Cdk4·cyclin D3 to start the $G_1$ buildup.[35]

The nuclear Cdk4·cyclin D kinases' job is to disable the Rb and the other pocket proteins and let E2F1's self-enhancing surge start, but only when the cell has reached the right size and it is ready to turn on the DNA-replication genes and build the replication factories on the nuclear matrix. When these kinases finally reach a certain level, they overwhelm the p21 and p27 CKIs and hyperphosphorylate various of the pocket proteins' 16 Ser and Thr residues (for example, by specifically targeting Rb's $Ser^{780}$ and $Ser^{795}$), which allows the E2F1 and E2F3 C-termini to slip out of the "tail-cuffs."[18] The D-type cyclins may also liberate the E2Fs from the pocket proteins by directly binding to the proteins' pockets, or Cdk4·cyclin D may knock an inhibitor such as Id2 off of E-box-bound E2F·DP complexes.[8,19,20] The liberated E2F·DP-1 heterodimers can then switch on the waiting RNA polymerase II transcriber and start the transcription of their target genes.[7,12,13,19,20] Thus, the Cdk4 or 6·cyclin D protein kinases link the cell's proliferogenic mechanisms to cues from the outside world and in response to these cues drive the $G_1$ buildup to the so-called *R (restriction) point* or *gate* where further progress becomes internally driven without input from growth factors. Thus, cutting off growth factor signals after the cell passes through the R gate does not affect the buildup to replication, but losing growth-factor-induced Cdk4/6·cyclin D activity before it can inactivate the pocket proteins and let the cell pass through the R gate prevents entry into the S phase.[11,35a]

When the accumulating nuclear cyclin Ds and the now active Cdk4·cyclin D protein kinases in the chromatin spill over the CKI barrier, they open the door to the replication-initiating gene expressions and other events by releasing the first few E2F1·DP-1s on the E2F1 and the cyclin E genes' promoters from the pocket protein's repressive grip. This starts E2F1 and cyclin E accumulating. While the transcription of the cyclin E gene has been low, any free cyclin E has been polyubiquitinated by a substrate-specific Cul3-dependent ubiquitin ligase and promptly consigned to the proteasome shredder to prevent its premature entry into action and disregulation of the $G_1$ buildup.[35b] But now as the cyclin E gene transcription surges upwards, the new protein is protected from the shredder and configured for joining Cdk2 by CCT, a cytosolic chaperonin.[35b] The Cdk2-ready cytoplasmic cyclin E is then promptly swept up by a cargo-carrier, importin, and whisked through the nuclear pores into the nucleus where it joins phospho-Cdk2 to produce a mid-to-late-$G_1$ surge of chromatin-associated inactive phospho-Cdk2·cyclin E kinases, which are being prevented by $p27^{Kip1}$ from being activated. The inactive phospho-Cdc2·cyclin Es are then activated when $p27^{Kip1}$ crashes and the liberated enzymes are promptly dephosphorylated and switched on by Cdc25A phosphatase (and maybe the Cdc25B phosphatase, which is expressed only near the end of the $G_1$ buildup[2])[8,25,33,34] that may be activated by a $Ca^{2+}$-dependent protein kinase (Fig. 1.2), which we will meet in the next chapter, as well as by the emerging active Cdk2·cyclin Es themselves.[35a] This sharp pulse of nuclear Cdk2·cyclin E activity is the much talked about *R-point factor* that starts the construction of the replication factories on the nuclear matrix.[35a,36,37] The importance of cyclin E in the $G_1$ buildup and the proteolytic control of its level for regulating proliferation is demonstrated by the fact that a cell overexpressing cyclin E doesn't need nuclear Cdk4·cyclin Ds to start the $G_1$ buildup.[37]

The normal intracycle CDK program requires that the Cdk4/6·cyclin Ds be silenced after they have inactivated the pocket proteins and started the escalating E2F and cyclin E

surges. This is done by the surging E2F·DPs stimulating the expression of the Cdk4/6-selective $p16^{INK}$ CKI.[19]

One of Cdk2·cyclin E's first jobs upon its arrival in the nucleus is to decondense the chromatin containing the replication-related genes in order to let transcription factors get at the genes' promoters.[37a] It does this by phosphorylating the nucleosome-interlinking H1 histones.[37a] The subsequent transcription of these genes is stopped by the remaining nucleosome aggregates, which are then loosened by another protein kinase, MSK1, which selectively phosphorylates the H3 histones in the nucleosomes' cores.[37a]

The surging nuclear Cdk2·cyclin E and E2Fs turn on genes in matrix-associated transcription factories that encode the proteins involved in replicating DNA.[36,38] One example of these genes is the thymidine kinase gene.[36] This gene's promoter has three domains: MT1, MT2, and MT3. The MT1 domain includes a perfect "GC" box (-96 CCCGCC-91), which is the binding site for the Sp1 transcription factor. The MT2 domain includes an E2F·DP-1-binding site, GTTCGCGGGCAAA. The MT3 domain binds the Egr-1 transcription factor. The Sp1 transcription factor attracts the basal RNA polymerase II transcribing complex consisting of the transcriber and its co-factors, TFIID and TFIIH (which is none other than our old CDK friend, CAK, a.k.a. Cdk7·cyclin H, complexed with MAT1(p36) to make it specific for the transcriber![23]), which starts transcription by phosphorylating the C-terminal domain (Tyr-Ser-Pro-Thr-Ser-Pro-Ser) of the polymerase's large subunit.[38] But this is held at the basal level by the E2F·DP-1·p107 pocket protein complex on the MT2 domain and the Egr-1·Rb complex on MT3. The cycle-starting signal causes an increase of Sp1, which cannot stimulate the gene's expression because of the blocked MT2 and MT3 domains. However, accumulating Cdk4·cyclin D1 protein kinase complexes target the Rb protein in the Egr-1 complexes on MT3. The hyper-phosphorylation of Rb removes the configurational block at MT3, but there is still no large surge of expression: the promoter needs more reconfiguration. This happens with the arrival of the Cdk2·cyclin E, which targets the pocket protein bound to the E2F·DP-1 complex on MT2. The hyperphosphorylated pocket protein is released, leaving an E2F·DP-1·Cdk2·cyclin E complex on MT2. Like the Sp1- recruited TFIIH/CAK·MAT1(p36), Cdk2·cyclin E triggers transcription by phosphorylating the C-terminal domain of RNA polymerase II's largest subunit. Thus, E2F·DP-1 has triggered a large burst of thymidine kinase production at exactly the right time in the program by bringing a CDK that is expressed only in late $G_1$ phase to the RNA polymerase II transcriber waiting with its engine idling in a pre-transcription complex.

The *DHFR* (dihydrofolate reductase) gene is regulated like the thymidine kinase gene. It too has Sp1- and E2F3(or E2F1)·DP-1-binding domains in its promoter.[38] As with the thymidine kinase gene, premature large-scale transcription by the rising level of Sp1 is prevented by an E2F·DP-1·pocket protein complex on the E2F·DP-1-binding domain. The triggering of its transcription along with thymidine kinase and the other replication-related genes in late $G_1$ phase requires the hyperphosphorylation and release of the pocket protein from promoter-bound E2F·DP-1·pocket protein complex by Cdk2·cyclin E, which then phosphorylates and thus switches on the waiting RNA polymerase II.

While these are examples of E2F-mediated stimulation of replication-driving gene expressions, an E2F isoform such as E2F3 collaborates with pocket proteins to *inhibit* the transcription of other proliferation-related genes such as B-*myb*.[17a] In the quiescent

$G_0$ cell, the E2F isoform is bound to its specific site on the B-*myb* promoter, but here it *inhibits* transcription by serving as the binding site for a p107 or p130 pocket protein, which in turn binds and stabilizes the binding of DRF (downstream repression factor) to an adjacent DRS (downstream repression site).[39] The E2F-dependent block is lifted late in the $G_1$ buildup when Cdk2·cyclin E phosphorylates and thus releases the p107/p130 from E2F. This causes DRF to lift off the DRS and expose the E2F·DP-1 complex, which relieves the repression and enables transcription to be started perhaps by an E2F·DP-1·Cdk2·cyclin E complex.[39]

The cyclin A gene has been silent until now because of the binding of an E2F-like factor to its promoter. This protein, like the E2F on the B-*myb* promoter, serves as the specific platform for a p107 or p130 pocket protein, which binds and thus holds a CHRF (cell cycle genes homolgy region factor) on an adjacent CDE (cell cycle-dependent element) suppressor site.[39] Here, too, the E2F-dependent block is lifted when Cdk2·cyclin E phosphorylates and releases the pocket protein from E2F. The release of the pocket proteins causes CHRF to lift off the CDE site, which activates E2F·DP and thus derepresses the cyclin A gene.

Cdk2·cyclin E does other very important things in its short, but busy, life that are needed in the later stages of the cycle long after it has gone. It stimulates the expression of the gene encoding the mitosis-starting Cdk1 (originally called Cdc2 after the fission yeast's CDK), which has also been suppressed until now by the same E2F·p107/p130·CHRF complex that was suppressing the cyclin A gene.[39] It may phosphorylate and thus hold back the APC (anaphase-promoting complex, which is not to be confused with another APC (adenomatous polyposis coli) that we will meet later on) that otherwise could selectively destroy the emerging mitosis-specific CDKs.[30] It also stimulates centrosome duplication (in animal but not plant cells) that will be needed to form the bipolar mitotic spindle after chromosome replication.[40–42] The elimination of Cdk2·cyclin E prevents further rounds of centrosome duplication, which would produce a multipolar spindle with a disastrous unequal distribution of chromosomes to the daughter cells.

Cyclin A, like its predecessor cyclin E, does not collect in the cytoplasm.[30] It too is promptly shipped from the cytoplasmic factory into the nucleus, but unlike cyclin E it has to be associated with a CDK to be imported into the nucleus.[33] The resulting surge of Cdk2·cyclin A into the nucleus shuts down Cdk2·cyclin E (Fig. 1.2).[11,36] Cdk2·cyclin A protein kinase displaces Cdk2·cyclin E from promoter-bound E2F·DP-1 complexes; it phosphorylates the cyclin E in the Cdk2·cyclin E complexes; the phosphorylated cyclin E is then polyubiquitinated by the Cul1-dependent SCF ubiquitin ligase and dumped into the proteasome shredder; and its Cdk2 partner is released to associate with cyclin A.[2,35b,43] Unlike Cdk2·cyclin E, Cdk2·cyclin A can bind to, and phosphorylate, E2F, which abolishes the ability of the E2F·DP-1 heterodimer to bind to DNA and continue promoting target gene expression, but it also takes over from Cdk2·cyclin E the job of keeping the pocket proteins in the inactive hyperphosphorylated state.[19,36] But as we shall soon see, Cdk2·cyclin A has other extremely important jobs in the DNA replication factories, one of which we will talk about below and the other of which is to hyperphosphorylate and thus activate B-Myb, which is somehow needed for the initiation of DNA replication.[39,44]

At this late stage in the $G_1$ buildup, the nuclear Cdk4/6·cyclin Ds are no longer needed and leave the chromatin for the nuclear matrix. To get rid of them, their cyclin Ds

are first phosphorylated by GSK(glycogen synthase kinase)-3β.[30] This causes them to be shipped out of the nucleus into the cytoplasm where their phosphorylated cyclin Ds are ubiquitinated possibly by the $SCF^{Cdc4}$-like complex and sent to the proteasome shredder,[33] or they are destroyed by calpain later in the cycle.[43]

However, before we go any further, we must try to find out why our cell needed to be attached by β1 integrins to the ECM (extracellular matrix) to get this far. The cell's ECM (substrate)-attached/activated β1 integrins have stimulated the membrane-associated ILK (integrin-linked serine/threonine kinase of which we will hear much more in Chaps. 3 and 4), which, in turn, has phosphorylated and inactivated GSK-3β.[45] If the cell had been detached from the ECM, and ILK silenced before reaching the R-gate, GSK-3β would have been active and would have phosphorylated cyclin D1 on $Thr^{288}$ and thus tagged it for prompt ubiquitination and consignment to the proteasome shredder.[2,45,46] Without Cdk4/6-cyclin D1 the cell could not have silenced the pocket proteins and started the surges of E2F, Cdk2-cyclin E, and Cdk2-cyclin A. Another contributor to anchorage dependence in some cells is the inhibition of members of a serine/threonine protein kinase family, the PAKs (p21-activated kinases), by a cyclic AMP surge and burst of PKA (cyclic AMP-dependent protein kinase) activity that is caused by detachment from the ECM.[46a] The loss of PAK activity results in the disassembly of cortical actin-based scaffolds for growth factor signaling complexes and an inability of the cell to even start building up to chromosome replication.[46a]

However, there would have been a much nastier consequence to a lack of attachment. An oncogene like c-Myc can actually kill its cell! c-Myc overexpression sets in motion a mechanism that will cause a cell to kill itself unless the survival-ensuring protectors accumulate normally once the $G_1$ buildup has started. The reason for this stems from there being too much E2F1. It has been suggested that E2F3 is the workhorse that stimulates the expression of the genes for the replication factories, while E2F1's job is to respond to DNA damage and uncontrolled proliferation.[17a] E2F1 is the only member of the E2F family that can stimulate the accumulation of p53(TP53), which stimulates both the expression of the $p21^{Cip1/WAF1}$ CKI and apoptosis (a.k.a., physiological cell death, programmed cell death, autogenous cell death) unless it is put out of action and degraded by binding to another protein, Mdm2, one of the protectors that operates during a normal $G_1$ buildup (Fig. 1.1).[47–51] (Mdm2 is a nucleus→cytoplasm shuttle that binds p53(TP53), and promotes its polyubiquitination, transportation out of the nucleus, and dumping into the proteasome shredder.[48a,52]) However, excessive E2F1 stimulates the expression of the $p19^{ARF}$ gene (an alternative reading frame of the gene for the $p16^{INK\,4a}$ CKI[48a]), the product of which protects p53(TP53) from Mdm2-mediated destruction.[48,48a] Also, there would not have been the PtdIns-3K (phosphatidylinositol (4,5)-3OH kinase) activity needed to make PtdIns(3,4,5)$P_3$ for stimulating the ILK and PKB/AKT kinases that stimulate the expression of the apoptosis-preventing Bcl-2 protector protein, inhibit the apoptogenic BAD protein, and suppress the apoptosis-driving caspase (cysteine aspartate-specific) proteases.[2,45,46] But something else would have been lacking because of the failure of the detached cell to phosphorylate and inactivate GSK-3β — a GSK-3β-promoted phosphorylation, ubiquitination, and shredding of β-catenin (of which we will learn much more in Chaps. 3 and 4), an activator of the LEF/TCF transcription factor that stimulates the expression of certain cell cycle genes such as those for cyclin D1 and c-Myc.[53,54] Therefore, not only couldn't the detached cell have initiated and completed a $G_1$

buildup, but with its excessive E2F1, p53(TP53), and unrepressed proapoptogenic proteins it would have suffered suspension-induced apoptosis or *anoikis*.[54,55] Of course, if the cell could somehow have maintained ILK activity and (or) hyperexpressed β-catenin while keeping GSK-3β suppressed without ECM-clustered/activated integrins and ILK, it would have had some of the most dangerous features of a cancer cell — an ability to proliferate without ECM attachment, avoid anoikis, and colonize foreign territories.

While the waves of cresting and crashing cyclin-dependent protein kinases are key drivers of the $G_1$ buildup, there is another family of protein kinases operating in these later stages of the $G_1$ phase — the PKAs (cyclic AMP-activated protein kinases). There is a lot of evidence for there being a transient surge of cyclic AMP in the middle to late $G_1$ phases of diatoms and budding yeast (*Saccharomyces cerevisiae*), lymphocytes, regenerating liver cells, cultured primary neonatal hepatocytes, thyrocytes, and the cells of various established lines.[13] This cyclic AMP transient is needed to initiate DNA replication.[13,14] The type I PKA is specifically employed in driving the cell cycle, and as we shall see further on, downregulation of the expression of the type I PKA regulatory subunit and upregulation of the expression of the type II regulatory subunit are parts of differentiation programs.[13,14,56–61] In TSH (thyroid-stimulating hormone)-stimulated rat FRTL5 thyroid cells and serum-stimulated MCF-10A human mammary gland cells, there is a large stimulation of RIα mRNA and production of RIα protein subunits, which peaks before the onset of DNA replication.[60,61] There is little or no increase of RII expression.[60,61] Preventing the RI surge with antisense oligodeoxynucleotide prevents or partially prevents the initiation of DNA replication.[56–61] The importance of the PKAs for the $G_1$ buildup has also been convincingly demonstrated by Sheffield, using mouse mammary cells and an oligonucleotide which was antisense to the translation-start site of the mRNA encoding the PKAs' common catalytic subunit.[62] In these cells, PKA activity rose fourfold to a peak shortly before the onset of DNA replication after quiescent cells were stimulated to start cycling by adding fresh fetal bovine serum to the culture medium.[62] Adding the antisense oligonucleotide along with the serum prevented both the surge of PKA catalytic subunits and the initiation of DNA replication.[62] Moreover, blocking PKA activity with the specific inhibitor, K5720, blocks human diploid dermal fibroblasts midway through their 13-h $G_1$ phase.[63]

What do the PKAs do in the $G_1$ buildup? For at least a quarter of a century, it has been believed that the PKAs are only inhibitory cell cycle regulators despite the very considerable evidence to the contrary.[13,64–66] All of the data point to a cyclic AMP *transient* and *pulse* of type I PKA activity driving some part of the later $G_1$ buildup.[13,64,65] However, a burst of PKA activity outside the critical time or a continuous elevation of the cyclic AMP inhibits the buildup, which is why cyclic AMP and the PKAs are widely believed to be cycle blockers.[13,64,65] At least one of cyclic AMP's jobs is to stimulate the expression of cyclin A and consequently the appearance of Cdk2·cyclin A, which replaces Cdk2·cyclin E and drives the replication of DNA in the factories set up in large part by Cdk2·cyclin E. Besides having an E2F-binding E-box, the cyclin A gene's promoter has a cyclic AMP-responsive element which binds CREB (cyclic AMP response element-binding) and CREMτ (cyclic AMP response element modulator) transactivators.[67–70] Reversible (by protein phosphatase 1 or 2A) phosphorylation of the CREB protein's $Ser^{133}$ and the CREMτ protein's $Ser^{117}$ in their glutamine-rich "P-boxes"

by PKA catalytic subunits surging into the nucleus converts the proteins bound to the CREs of target genes' promoters into powerful transcription activators.[68] In quiescent $G_0$ and early $G_1$ phases, human diploid fibroblasts, the phosphorylated CREB and CREMτ proteins on the cyclin A gene's promoter, are inhibited by heterodimerization with ICER (inducible cAMP early repressor, a truncated CREM gene product[68]).[67] However, the ICER inhibitor transiently crashes near the end of the $G_1$ buildup, which makes the cyclin A gene *briefly inducible* by the late $G_1$ phase cyclic AMP surge and burst of type I PKA activity, which, incidentally, may also be responsible for the necessary downregulation of cyclin D gene expression.[67,71]

As we learned above, the cyclin A takeover in late $G_1$ phase involves the polyubiquitination of phosphorylated cyclin E by Cul1-dependent ubiquitin ligase.[35b] PKA may be involved in this by stimulating the expression of ubiquitin hydrolase, which would promote the shredding of cyclin E by the proteasome.[72] However, while getting rid of cyclin E and, of course, CKIs, such protein destruction must be sufficiently selective to avoid destroying the CDKs and allow the buildup of key replication components such as the M1 subunits of ribonucleotide reductase.[73–75]

However, the cyclic AMP-activated type I PKA does not only stimulate the cyclin A gene. It also stimulates the delivery of various transcription factors and other proteins, including cyclin A, into the nucleus and mRNAs out of the nucleus.[76–82] Indeed, the late $G_1$ cyclic AMP and cyclin E (Cdk2·cyclin E) transients mark the time when agents such as bFGF, a stimulator of ribosomal RNA transcription (and therefore protein synthesis), and the newly made replication-related components such as cyclin A are carried by a stimulated transport machinery through the nuclear pores into the nucleus.[37,83] Thus, it appears that the burst of PKA activity caused by the mid to late $G_1$ cyclic AMP transient stimulates the cyclin A gene (because the ICER inhibitor has temporarily crashed) and at the same time might open nuclear pores and increase the activity of the nuclear pore machinery[84,85] that exports the activated cyclin A gene's messages into the cytoplasm for translation and then imports the freshly minted Cdk2·cyclin A (along with other components such as the NF-κB transactivator[80]) into the nucleus to help start DNA replication.

The cyclic AMP surge may also be part of the mechanism that together with PKB/AKT signals from adhesion-activated β1-integrins suppresses the E2F1/p53-mediated apoptogenic arm of c-Myc action. The cyclic AMP surge activates PKA attached by AKAP (A-kinase-anchoring protein) to mitochondria.[86] The activated PKA then phosphorylates BAD protein's $Ser^{112}$, which inactivates this apoptosis-promoting protein.

## A checkpoint to pass

*"We know that cells have a well-organized set of molecular controls that govern their progress through the cell cycle. In particular, there is a series of 'checkpoints' that each must successfully negotiate before it can proceed to division. Indeed, it would be surprising if things were otherwise, for division is quite the most taxing and dangerous task that the cell must perform.*

*The situation is like a passenger jet taxiing to the runway for take-off. The crew must run through a long list of pre-flight checks before they commit themselves and their*

*passengers to hurtling down the runway at breakneck speed in the hope of soaring gracefully aloft before they reach the end."*

— T. Kirkwood[87]

As indicated in Fig. 1.1, there is a checkpoint towards the end of the $G_1$ buildup when the cell checks its chromosomes to see whether they are fit for replication. Checkpoint mechanisms are not essential for cell cycle transit, but when activated they can pause the buildup to allow chromosomal repair and thus promote genomic stability. The principal players in this first checkpoint are a member(s) of the family of DNA-activated protein kinases (ATM [ataxia telangiectasia] protein kinase and DNA-PK) and p53(TP53).[88-96] Indeed, Rensberger[97] has described p53(TP53) as "... *a quality control agent with responsibility not to its own cell but to the republic of cells.*" It has been generally believed that it is the DNA-PK that detects and signals the presence of damaged DNA, but ATM can "stand in" for DNA-PK and may in fact be the more important sensor because fibroblasts from DNA-PK-null *scid* mice still activate p53(TP53) in response to DNA damage.[98]

Underphosphorylated, active p53(TP53) accumulates near the end of the $G_1$ buildup, and phosphorylation of $Ser^{315}$ near its nuclear localization sequence by one of the $G_1$ Cdk-cyclins, possibly Cdk2-cyclin E or A, stimulates its transport into the nucleus to carry out its damage-checking mission.[1,13,99] If chromosomes have been broken, DNA-PK or ATM, kinase will be activated by binding to the exposed double-stranded ends of broken chromosomes, or the single strands in large gaps.[13] Either switched-on kinase then phosphorylates $Ser^{15}$ in p53(TP53)'s N-terminus and at the same time phosphorylates and turns on a second kinase, CHK2, that phosphorylates p53(TP53)'s $Ser^{20}$.[99a] These two phosphorylations activate p53(TP53) and prevent it from being degraded by binding to Mdm2.[48,89,99a] p53(TP53) will also be activated by the mismatch repair mechanism responding to gene mutations without double strand breaks.[91] However, p53(TP53) can also somehow be activated without DNA strand breaks by hypoxia and depletion of ribonucleoside triphosphates.[93] The activated p53(TP53) then starts a flurry of events meant first to stop the initiation of DNA replication to buy time to repair damage, but also, if the damage be excessive and (or) irreparable, to trigger apoptosis and kill its cell for the good of the cellular society as a whole. It must be noted that while checkpoint mechanisms in the single-celled bacteria and yeast are aimed only at the cell's own survival, the priority in a multicellular organism is the elimination of mutant cells that would give rise to clones of cells (e.g., malignant cancer cells) that could destroy the organism. Indeed, without this shift in priorities multicellular organisms could not have arisen.

To stop the onset of replication, the activated p53(TP53), with its $Ser^{15}$ and $Ser^{20}$ residues phosphorylated, stops the buildup to DNA replication, first by stimulating the expression of the *mda*-6 gene, the promoter of which has two p53(TP53)-recognizing elements and the product of which, the $p21^{Cip1/WAF1}$ CKI, inhibits Cdk4-cyclin D and Cdk2-cyclin E kinases and, with less affinity, Cdk2-cyclin A.[93,99,100] $p21^{Cip1/WAF1}$ also directly reduces or stops DNA strand elongation by directly binding to PCNA, which as we shall soon see clamps onto a DNA template strand and serves as sleeve or platform to guide the strand's processive replication by DNA polymerase δ in the replication factories.[100] Of course, the polymerase needs deoxyribonucleotide building blocks to

make new DNA repair patches. The doubly phosphorylated p53(TP53) provides these blocks by stimulating the expression of a repair-specific ribonucleotide reductase gene, *p53R2*.[100a]

If the damage is great and deemed irreparable, p53(TP53) will proceed to trigger the lethal apoptogenic mechanism with the products of other genes.[93,99a,101,102] But how does p53 know when to just delay the onset of replication and repair the damaged DNA or regard the damage as irreparable and trigger the apoptogenic mechanism? The answer lies in the phosphorylation of p53's $Ser^{49}$ residue. If the shock and the damage it inflicts should exceed a certain level, another protein kinase ($PK^{Ser49}$) kicks into action and reconfigures $p53^{PhosSer15,20}$ by phosphorylating $Ser^{49}$. The reconfigured $p53^{PhosSer15,20,49}$ now binds to, and activates, the promoter of the gene coding for p53AIP.[102a] p53AIP then goes to the mitochondria and triggers the lethal apopoptogenic cascade by causing the formation of the huge mitochondrial permeability transition pore that we will meet in Chap. 5.

Standing in the way of carrying out this death sentence are the anti-apoptosis Bcl-2 protector protein (more of which in the next chapter) and the survival or rescue signals from the receptors for autocrine/paracrine IGF-I.[101-102,103-105] But p53(TP53), taking no chance that the dangerously defective cell might survive, sweeps these aside by shutting down the Bcl-2 gene, activating the gene encoding the apoptosis-promoting Bax protein (which causes an apoptogenic release of cytochrome c from the mitochondria into the cytoplasm), and inducing the expression and secretion of active IGF-BP3 (IGF-binding protein 3), which silences the IGF-I receptor signals by binding autocrine/paracrine IGF-I.[93,99,99a,103]

However, if there has been nothing to attract the attention of DNA-PK, ATM, or the gatekeeper, then chromosome replication will start (Fig. 1.1).

## The S phase: replicating the chromosomes — a daunting task

When the buildup reaches a climax, replication enzymes, histones, and other components start flowing into the nucleus and collecting at replication factory construction sites. At the beginning of the $G_1$ buildup, there already had been an ATP-driven binding of six-component ORCs (origin recognition complexes) to the origins of the set of "housekeeping" genes that will be the first to be replicated.[12,106,106a] Then, early in the buildup the 62-kDa Cdc6 protein ($p62^{Cdc6}$ in human cells) appeared and associated with the ORC replication "launching pads," which paved the way for the attachment of six pro-helicase MCM (minichromosome-maintaining) proteins to the complexes by a mechanism requiring the association of the MCMs with RLF-B (replication-licensing factor-B) factor.[12,26,106-108] The resulting complexes were the pre-replication complexes which were "licensed" for replication.[12,106] Now, at the end of the buildup, these licensed complexes are the targets of the Cdk2·cyclin As resulting from the PKAI-driven expression and nuclear import of cyclin A. The Cdk2·cyclin As stimulate the replacement of Cdc6 by Cdc45 to form ORC·Cdc 45·MCMs pre-initiation complexes.[12,106,106a] Now everything is ready for the final activation of the "Master of Ceremonies" helicase that starts chromosome replication in the first replication factories using components such as the histones that have resulted from the E2F/Cdk2·cyclin E activities (Fig. 1.2) and been shipped into the nucleus by the cyclic AMP/PKAI-stimulated nuclear import

mechanism. The deoxyribonucleotide building blocks for replicating DNA are fed directly into the replication factories from associated large enzyme complexes.[108a]

At the end of the $G_1$ buildup, cyclin-like Dbf4p (or ASK in humans) proteins appear alongside cyclin A, which bind to the pre-initiation complexes.[109] Each of these proteins then attracts a Cdc7 (huCdc7 in human cells, muCdc7 in mouse cells) protein, which becomes the catalytic subunit of Cdc7·Dbf4p(ASK), a DDK serine/threonine protein kinase.[109] This DDK kinase phosphorylates MCM proteins, some of which leave the pre-initiation complex while the remaining three types of MCM (MCMs 4, 6, and 7) on Cdc45 become an active, six-membered helicase ring (consisting of two of each of the three MCMs), which by clamping around the DNA strands and separating them enables replication to start.[12,106–110] However, once they have been pulled apart by the DDK-phosphorylated MCMs·Cdc45 complex, the separated origin single strands must be held apart. This is the job of hyperphosphorylated RP-A.[110–112] The first step in the production of hyperphosphorylated RP-A was the phosphorylation of its 34-kDa subunit by Cdk2·cyclin A, which was followed by further phosphorylation by DNA-PK (DNA-dependent protein kinase), which was activated by binding to the now accessible single DNA strands.[110–113] This causes us to pause and note that Cdk2·cyclin A doesn't just fade away like Cdk2·cyclin E after it has helped start the first wave of replications. Instead, it continues to work hard in the replication factories throughout the S phase, helping to make pre-replication complexes and then activating RP-A in the successive "firings" of batteries of replicons (a replicon: a stretch of DNA whose replication is controlled by one origin).

By the end of the $G_1$ buildup, enormous (17S–18S) replication factories composed of synthesomes, each having a core of about 35 replication enzymes (about half of which are poly[ADP]ribosylated) and their associates (DNA polymerases $\alpha$, $\beta$, $\delta$, $\epsilon$, [Pols $\alpha$, $\beta$, $\delta$, $\epsilon$], DNA ligase 1, DNA primase, PARP [*p*oly[*A*DP]*r*ibose *p*olymerase], PCNA (proliferating cell nuclear antigen), the RF-C (replication factor-C) clamp loader, topoisomerase II, and, of course, Cdk2·cyclin A) start working to replicate first the essential "housekeeping genes."[110,114] Even more enormous factories will then be built to replace the first factories in order to replicate the genes for differentiation-related products and last of all the heterochromatin-cloistered inactive genes.[114] These factories are located in replication foci attached to the nuclear matrix where there are clusters of replicons (stretches of DNA, the replication of which is controlled by one functional origin), each of which is served by one synthesome.[110,114,115] The copying of a DNA template strand by the core synthesome is an extremely complicated, seemingly cumbersome, yet astonishingly error-free, process, which we will try to briefly outline. It seems that replication of a replicon involves the attachment of the DNA double strand to the factory by its origin, pulling it into one end of the factory, its separation into two template strands, and finally extrusion of the replicated daughter strands from the other end.[114] In other words, the polymerases and their large retinues do not track along the DNA strands like mini-locomotives. Instead, the strands are pulled through them using the energy released from the hydrolysis of the deoxyribonucleoside triphosphates during their incorporation into new strands.[114]

Now let us look into one of these factories and watch a replicon being pulled in and replicated as it moves along a synthesomal assembly line. After the double-stranded origin DNA has been separated ("melted") by the MCM helicase ring, and the

hyperphosphorylated RP-A protein has bound to the 3′→5′ single strand, the RP-A protein is grabbed by a waiting DNA polymerase-α-associated primase, which makes a short RNA copy/primer for what will be the 5′→3′ copy (the so-called "leading strand") of the strand.[106a] As the strand moves along, driven by the puffs of energy released with each nucleotide addition, the primer is then extended by Pol-α only for a short distance before it is snipped off at the next stop on the assembly line by a waiting RNAse (RNAses H and FEN/RTH 1) and then replaced with DNA by Pol-ϵ. While this is going on, the RF-C clamp loader has detected the passing primer's terminus. It reacts to this by knocking Pol-α off RPA, then binding to RP-A and organizing the assembly of PCNA subunits into a homotrimeric ring that clamps itself around the DNA strands.[106a,116] The PCNA ring then binds to the next polymerase, Pol-δ, in the process, knocking RF-C off RP-A. The PCNA ring then serves as a sliding clamp that keeps the attached Pol-δ working processively as the template strand is pulled by or through it.[106a,116] Of course, before a single template strand can be ringed by PCNA and replicated by Pol-δ, the double-stranded DNA must first have been untangled by the ring-shaped topoisomerase I and melted by helicase I or IV.

It is more difficult to make the 3′→5′ copy (the so-called "lagging strand") of the 5′→3′ template strand, because the synthesome won't add nucleotides in the 3′→5′ direction. Therefore, things must be arranged so that the synthesis can be done in the 5′→3′ direction. This is done by replicating the strand in pieces called "Okazaki fragments," named after their discoverer.[117] First, the passing 5′→3′ strand is cut to expose a 3′ end, and the Pol-α-associated primase makes the required short 5′→3′ RNA primer. Pol-α adds a short stretch of DNA to the primer. The RNA part of the primer is snipped off and replaced with DNA by Pol-ϵ. Of course, there is now a 3′ primer terminus onto which RF-C loads a PCNA clamping ring. Pol-δ attaches to the ring, and the passing template strand is replicated by PCNA·Pol-δ until it hits the 5′ head of an adjacent fragment on the passing template. At this juncture, RF-C kicks PCNA and Pol-δ off, and the head-to-tail fragments are tied together into a 3′→5′ strand by the assembly line's DNA ligase 1. Then, the 3′→5′ replication must be finished by capping the ends of the extruding strands with a common telomere sequence to prevent a dangerously "sticky" end and a loss of genetic material. However, that is another story for another book.[117a]

There is only one round of DNA replication in a normal cell cycle. But how can further rounds be stopped when the nucleus is loaded with replication factories and foci? The explanation lies with Cdc6, the pre-replication complex former that was displaced from ORC and replaced with Cdc45 by Cdk·cyclin A. Cdc6 is gone: it will not reappear in this cell. It will appear only in the daughter cells.[12,12,106] Even if by chance it did reappear, the mitotic CDKs that will replace the soon-to-crash Cdk2·cyclin A would phosphorylate it and prevent the reassembly of a pre-initiation complex. Therefore, a second round of replication could not begin until the destruction of the mitotic CDKs at anaphase. As if this were not enough, phosphorylation by Cdk2·cyclin A also prevents the reassociation of the DDK-displaced MCMs with freshly replicated origins during the S phase.[11,12,106] Finally, while the displaced Cdk2·cyclin A-phosphorylated MCMs stay in the nucleus, RLF-B, the licensing factor that would enable them to associate with the pre-initiation complex (if they could) and be pulled into a factory, is lost, and any new cytoplasmic RLF-B that might be lurking in the cytoplasm could not get at the origins until the nuclear membrane breaks down at mitosis.[11,12,26,106]

The daughter cells and their chromosomes do not emerge functionally *tabulae rasae* like the cells in eight-celled embryos and blastulas. The mother cell's functional characteristics are replicated with her genes as they are passed along the factory assembly lines: the genes on the daughter strands are correspondingly marked for expression or suppression. This is done in part by sticking a set of do-not-read Post-it® notes (to use Kirkwood's happy analogy[118]) onto the genes in the daughter strands that corresponds to the set of Post-it® notes on the genes of the parent strands. Most of the Post-it® notes are in the non-transcribed (inactive) DNA and late-replicating DNA. They are methylated cytosines located in the promoters of various genes. If these methylated cytosines are located in the attachment sites for transcription factors, they can permanently lock a gene into the "off" configuration.[119–122] The $CH_3$ groups are plucked from S-adenosyl methionine by DNA-MTase (5-cytosine DNA methyltransferase), which is attached to PCNA and looks for hemimethylated sites in the passing newly replicated DNA strands, and when it finds methylated cytosines in a template strand, it attaches methyl groups to the corresponding cytosines in the daughter strand.[119,121] About 50 000 of our genes (i.e., about one half) have large (0.5–5-kilobase) CpG/GpC islands in their regulatory regions. These are the so-called "housekeeping genes," and except for the islands of the genes on the inactivated X-chromosome and "imprinted" (i.e., selectively methylation-silenced paternal or maternal) genes, their islands are somehow protected from methylation (possibly by specific non-histone proteins). If not, there could be dramatic functional changes, which could include the silencing of CKI genes, which would disable proliferative suppression mechanisms in the daughter and, because of DNA-MTase, her progeny.[119,122,123] Indeed, the rising DNA-MTase activity during carcinogenesis is associated with the methylation of CpG-island "hot spots" and the shutdown of genes such as the $p16^{INK\,4a}$ CKI, a replicable "epimutation" (the gene itself is unaltered), which results in Cdk4-cyclin D hyperactivity, which disables the cycle-suppressing and tumor-suppressing Rb-family of pocket proteins.[119,122]

## Mitosis and the birth of two new cells

On the threshold of mitosis, the sister chromatids are tied together by tethering proteins known as cohesins that were installed during replication. Also during replication the chromosomes had become entangled or catenated at the points where the replication forks had collided. While most of these tangles have been removed by this time by topoisomerase II to enable the chromosomes to be separately and accurately condensed, some remain and will have to be removed during mitosis by the topoisomerase to allow the sister chromosomes to separate at anaphase.[123a]

One of best hidden facts of cellular life is the $G_2$ cyclic AMP transient and burst of PKA activity that is probably triggered by the last of the CDKs in a variety of cells, such as blastema cells in the regenerating annelid worm *Owenia fusiformis*, Chinese hamster ovary cells, fetal rat liver cells, HeLa human cervical adenocarcinoma cells, human RPMI 8866 lymphoid cells, rat kidney tubule cells, androgen-activated seminal vesicle cells, and *Tetrahymena pyriformis* as well as in pig, rabbit, and sheep oöcytes just before the onset of meiotic prophase.[13] The probable importance of this transient is indicated by the fact that although keeping a high intracellular cyclic AMP concentration in pig oöcytes prevents meiotic prophase, a transient surge of cyclic AMP and PKA activity

accelerates meiotic prophase.[13] One of the cyclic AMP surge's jobs is possibly to stimulate the expression of ubiquitin hydrolase as well as prevent the large, anaphase-promoting, ubiquitin-ligating complex/cyclosome, APC (which the reader will remember must not be confused with the APC tumor suppressor we will meet often in Chaps. 3 and 4 — the adenomatous polyposis coli protein disabling of which starts carcinogenesis in colon and other tissues), from stopping the buildup to mitosis by prematurely polyubiquitinating and delivering various things needed for prophase and metaphase to the proteasome shredder.[30,124–126]

The last of the cycle-driving cyclins are the mitotic cyclin Bs, which cannot mate with Cdk2 that rules the S phase.[11,13,14] However, the expression of their partner, Cdk1, has been suppressed by Cdk2·cyclin A during the S phase, but it appears when Cdk2·cyclin A crashes at the end of the S phase.[13,14] But cyclin A is still left in the nucleus.[13] It peaks in early prophase and joins with the now-accumulating Cdk1 to produce Cdk1·cyclin A kinase, which drives the initial events of mitosis. However, it will soon crash (by being phosphorylated and thus marked for destruction by the ubiquitination/proteasome mechanism) and leave the field to cyclins B1 and B2 from mid-prophase to the anaphase-GO! signal.

Until now, the Cdk1·cyclin B1/B2 kinases and the cyclin Bs have been shuttling to and from the nucleus with the nuclear export mechanism working hard to keep their nuclear level below a critical, prophase-triggering, threshold.[2,33,43] At some point, the export mechanism starts failing and Cdk1·cyclin B kinase complexes begin accumulating in the nucleus where they are activated and take over from Cdk1·cyclin A to finish the mitotic job (Fig. 1.2).[33] The first event leading to the formation of inactive Cdk1·cyclin B complexes is the phosphorylation of Cdk1's $Thr^{161}$ by the now familiar CAK, which enhances the binding of cyclin A or a cyclin B to Cdk1's PSTAIRE (Pro[*P*]-Ser[*S*]-Thr[*T*]-Ala[*A*]-Ileu[*I*]-Arg[*R*]-Glu[*E*]) motif-bearing region to form the holoenzyme.[13] Then, the Cdk1 kinase subunit is inactivated by having its $Thr(T)^{14}$ and $Tyr(Y)^{15}$ phosphorylated by a dual-function (or S/TY) protein kinase called "wee-1."[12] The suppression of Cdk1 expression until the end of replication and then the suppression of the Cdk1·cyclin enzymes' activity until the prophase starter signal (the identity of which will be revealed in the next chapter, although the reader can probably guess what it is) is given and makes sure that mitosis will not be aborted and the cell damaged or more likely killed by premature nuclear envelope breakdown and chromosome condensation.

Also to prevent a catastrophically premature prophase, the cell has another cycle-stopping checkpoint device that monitors the level of unreplicated DNA and is activated only if all of the chromosomes are not replicated (Fig. 1.1). A key part of this device is the 45-kDa RCC1 protein, a potent guanine nucleotide exchange (GTP for GDP) promoter for the Ran/TC4 nuclear transport G-protein, which is also associated with chromosome centromeres and is an essential part of replication complexes, which is obviously an ideal place from which to monitor the amount of unreplicated, single-strand DNA.[127–141] RCC1 prevents the premature initiation of prophase by maintaining Ran/TC4 in the active GTP·Ran/TC4 form which keeps Cdk1·cyclin B and the cyclin Bs shuttling in and out of the nucleus.[137] This mitosis-restraining role of RCC1 is dramatically demonstrated by the premature, although incomplete, mitosis that follows the destruction of the thermolabile mutant RCC1 in S-phase *ts*BN2 hamster cells by incubating the cells at the non-permissive 39°C.[136,138] Cells must destroy RCC1 by ubiquitin-mediated proteolysis

after replicating their chromosomes in order to initiate mitosis.[142,143] The GTP·Ran/TC4 complexes that have been sustained by RCC1 and have been preventing Cdk1·cyclin B kinases from accumulating in the nucleus are converted by the Ran-GAP GTP-ase enhancer into inactive GDP·Ran/TC4 complexes, which stops or reduces cyclin B export.[135,140] Now another key player, Cdc25C, a $G_2$/M-specific dual-function protein Ser/Thr/Tyr phosphatase, which has been waiting at the nuclear periphery, is activated by our soon-to-be-identified mitosis starter[144] and transported into the nucleus where it activates Cdk1·cyclin B kinases by dephosphorylating the $Thr^{14}$ and $Tyr^{15}$ in Cdk1's catalytic domain.[13,14,139] The mitotic CDKs phosphorylate H1 histones associated with internucleosomal ("linker") DNA, H3 histones in the nucleosome cores, and the 13-S condensin.[13,14,145] H1 histone superphosphorylation reconfigures the chromosomes to enable phospho-13S condensin to gain access to its "receptor" — phospho-H3.[14,145] Phospho-condensin then starts the ATP-dependent supercoiling that is the basis of chromosome condensation.[145]

The Cdk1·cyclin kinases trigger the production or activation of a clathrin-like vesicularization factor that breaks the nuclear envelope into vesicles and in the process dismantles the nuclear pore complexes.[13,14] The kinases also phosphorylate and directly depolymerize, or prime the depolymerization of, the lamina network that lines the inner surface of the nuclear envelope.[13,14]

While the chromosomes are condensing, the centrosomes are collecting $Ca^{2+}$-loaded vesicles around themselves and separating to form the bipolar spindle using BimC/Xklp2p-family KRP (kinesin-related protein) motors, and CEPs (centromere-binding proteins) and other motor proteins such as the CENP-E and MCAK KRPs start collecting on the centromeres, which are long stretches of highly repeated CEN base sequences organized into several microtubule-binding segments separated by linker segments.[13,14,145a] Eventually, mature trilaminar kinetochores will be assembled on the centromeres to collect a pack of motor and other proteins and the microtubule attachment devices that serve as handles for dragging the chromosomes toward the spindle poles at anaphase.

Tubulin pools are now redirected to making the mitotic spindle, and new dramatically stabilized microtubules (spindle fibers) start extending from the centrosome. The plus ends of the unstable microtubules growing from the centrosomes probe the cytoplasm, looking for the chromosomes' centromeres and their microtubule-grabbing kinetochores. When they find one, as many as 30 or 40 of them may clamp into the motor's several microtubule attachment sites where they are stabilized by the so-called $Ca^{2+}$-binding STOP protein.[13,14] Now they must line up along the mid-zone of the spindle, a process known as *congression* which is driven by the CENP-E motors.[145a] Other microtubules that do not find a kinetochore join side-by-side with microtubules growing from the opposite centrosome to form stable interzonal spindle fibers.

Now we have arrived at the last checkpoint — the SAC or Spindle Assembly Checkpoint (Fig. 1.1). Something monitors metaphase congression and prevents the initiation of anaphase until every pair of sister chromosomes has taken its place on the spindle and their kinetochores have attached to microtubules. The sister chromatids are still stuck together by the cohesin tethers that were applied in the S phase during their passage through the replication factories in order to keep them together until anaphase[145,146] as well as by their still undecatenated centromeric DNA. Those that have attached to the

spindle are waiting for the starting signal, straining in a state of dynamic tension from a tug-of-war between the sisters' kinetochores pulling toward opposite poles.[3,5,13,147] The starter signal they are waiting for is being suppressed by a limitedly diffusing signal emitted by the kinetochores of those pairs that have not yet attached to the spindle and been put under tension.[3,123a,142,148] In the simplest possible terms, the assembly of a set of so-called Bub and Mad proteins on unattached, unstrained kinetochores includes the Bub1(Mps1; 3F3/2 epitope) protein kinase that recruits and phosphorylates Mad2 protein, which then binds to local APC·p55CDC(Cdc20) complexes and prevents them from starting anaphase.[148] When microtubules are tethered to the kinetochore by the CENP-E and MCAK KRP motors, the combination of microtubule attachment and tension shuts off the protein kinases and kicks Mad2 and its APC-like accomplices off the kinetochores into the cytoplasm.[148] When all of the chromosomes have been attached and their kinetochore signaling silenced, the now unsuppressed spindle-associated APC·p55CDC-(Cdc20) can start anaphase when activated by the Anaphase-GO! signal.

The p55CDC(Cdc20) protein that has been accumulating since late in the S phase combines with APC to make the functional APC·p55CDC(Cdc20) complexes that will start anaphase when activated by a protein kinase, Plk (Polo-like kinase), after the expulsion of Mad2.[124–126,148] Until now, the cohesins that are tying the sister chromatids together have been untouched because the separin (Esp1) scissors for cutting these tethers have been locked up by securin (Pds1/cut2).[123a] The Anaphase-GO! signal must unlock the cohesin cutters. It must also get rid of the mitosis-starting Cdk1·cyclin B that up to this point has been driving the condensation of the chromosomes, otherwise the untethered sister chromatids will separate all right, but they will not decondense to form new daughter nuclei.[5,13,14] But before anything can happen, the APC·p55CDC(Cdc20)-restraining PKA activity also must drop to enable APC·p55CDC(Cdc20) to be phosphorylated and activated by Plk, which has been stimulated by Cdk1·cyclin B.[124,125] However, it seems that although the cyclic AMP/PKA surge inactivated APC·p55CDC(Cdc20), it also activated the proteasome that will be ready to shred the polyubiquitinated components when they are produced by the Plk-activated APC·p55CDC(Cdc20).[75,149] In the next chapter, we will learn that the calmodulin-dependent protein kinase II (CaMK II) is the prime mover of Cdk1·cyclin B destruction.[150]

One of APC·p55CDC(Cdc20)'s targets for polyubiquitination and consignment to the proteasome is the securin that has been restraining separin, the cohesin cutter.[123a,145,146] However, cutting cohesin tethers is not enough for chromosome separation — there are still the tangles holding the sister chromosomes together at their centromeres that must be removed by topoisomerase II.[5,14,123a,151,152] Cyclin B is targeted for polyubiquitination by APC·p55CDC(Cdc20) because of its *N*-terminal D (for destruction) box.[2,5,13,14] The Cdk1 liberated from Cdk1·cyclin B by the destruction of its partner cyclin B is inactivated (it becomes unable to bind cyclin A or B) by having its $Thr^{161}$ (which was phosphorylated by CAK) dephosphorylated.[2,13,150] Once the sister chromosomes are untethered and the CDK has been destroyed, the "Pac Man" motors of the chromosomes' kinetochores are switched on and the kinetochores start chewing their way up the spindle fibers to the spindle poles, each dragging its cargo of genes and other things behind it.

The APC activation and the exit from mitosis is ultimately triggered by Cdk1·cyclin B, which is thus the agent of its own destruction.[5,150] But how does the cell stop Cdk1·cyclin B from doing this too early and abort the proper packaging of chromosomes

for assembly on the metaphase spindle? Of course, one of the ways it does this is to cause a burst of APC·p55CDC(Cdc20)-inhibiting PKA activity.[145] Also, this may be one of the jobs of its prophase-peaking co-worker and immediate predecessor, Cdk1·cyclin A, before the cyclin A crash just before metaphase (Fig. 1.2). Cdk1·cyclin A does not trigger its own destruction and it prevents Cdk1·cyclin B from triggering the APC-cyclin-destroying mechanism through activated Plk.[150]

During chromosome replication along the assembly lines of the replication factories, the pre-replication complexes were dispersed when phosphorylated by the Cdk2·cyclin A kinases in the synthesomes. While the ORC complexes immediately re-attached to the duplicated origins, the MCM and the unstable hyperphosphorylated Cdc6 proteins could not reattach and were then discarded. No replacements could get into the nucleus. This prevented reinitiation and multiple rounds of DNA replication.[106,107] When the chromosomes have been ratcheted up the chromosomal microtubule bundles to the opposite poles (anaphase A) by their microtubule-depolymerizing Pac-Man-like MCAK kinase/motors, and the spindle poles have finished being pushed apart by the sliding of the interzonal spindle fibers along one another driven by MKLP1/CHO1 KRP motors lodged in the spindle midbody (anaphase B), the daughter cells enter telophase when they decondense their chromosomes and make new nuclei.[13,14,145a,148] It is around this time that they can re-license their chromosomes for replication. The nuclear membrane has not yet been assembled, the Cdk1·cyclin B kinases have long since gone, and unphosphorylated Cdc6 protein level has peaked.[106–110] Cdc6 can now associate with the potentially functional origins as they re-emerge in the decondensing chromosomes. The new nucleus now has the RLF-B1 replication licensor, which has been in the mother cell's cytoplasm but could not get through the intact nuclear envelope. The new cell will be able to replicate its chromosomes *if* it is signaled to start a cycle.

By anaphase the cell has wrapped a wonderful device around its middle just underneath the plasma membrane — a contractile belt of actomyosin fibrils, which has been kept relaxed until the right moment by having certain residues of the light chains of the myosin motor heads phosphorylated by Cdk1·cyclin B.[13,152a] However, this stops when the kinase is destroyed by the APC-exit mechanism and the inhibitory phosphates it planted on key residues of the myosin motors are removed by a surge of protein phosphatase activity. Then, a small G-protein, Rho, is activated during anaphase by being converted by a guanine nucleotide-exchanger from GDP·Rho to GTP·Rho and starts the myosin motors by enhancing the phosphorylation of another set of myosin light chain residues by calmodulin-dependent protein kinase II by inactivating myosin light chain phosphatase.[13,14,150] As the myosin motors pull on the actin filaments, they tighten the belt which guides vesicles for new membrane production into the cleavage furrow the belt produces as it pinches the large, elongating cell into two, usually normal size, daughters.[152a] The fate of the daughter cells will depend on the genes they are expressing (i.e., their transcriptome), the signals they receive from their neighbors, and the hormones and other factors flowing into the extracellular matrix from the blood (Fig. 1.1). If the daughters are normal, their Rb-family pocket proteins were dephosphorylated and reactivated during mitosis, which will prevent the initiation of a $G_1$ buildup until growth factor signaling resumes. If the replication-related genes are now locked shut, the cells may differentiate, work for a while, and then kill themselves. If their chromosomes are not licensed for replication in the replication factories, they will be unable to proliferate

regardless of whether or not their proliferogenes are locked shut. Could this be why mature neurons can function for decades without proliferating despite often intense signaling by neurotrophic and other factors that would start a $G_1$ buildup in other cells?

The neurons in the brains of the victims of Alzheimer's disease, choking with neurofibrillary tangles and nesting among clumps of β-amyloid protein, the perilously poised neurons in the penumbra of a stroked brain, and other crippled neurons of other neurodegenerative disorders bear witness to the terrible things that happen when proliferatively disabled cells respond to damage signals by trying to proliferate to replace lost cells and repair their damaged tissue.[153–164] While a tiny few neurons might succeed,[165] most try and *die*! Their abortive attempt to start a cycle results in the disastrously piecemeal expression of only some of the CDKs or their components such as cyclin D1, cyclin E, and cyclin B1, Cdk1, Cdk4, Cdk5 as well as replication components such as PCNA and the nuclear Ki-67 protein. The cell might even try to go directly into mitosis by expressing the mitotic CDK, Cdk1·cyclin B1.[163] One consequence of this is the appearance of the Cdk1·cyclin B1-phosphorylated tau protein of the neurofibrillary tangles of Alzheimer neurons, the phospho-epitopes of which are the same in mitotic cells.[163] However, since the stimulated neurons cannot replicate their chromosomes, the appearance of CDKs such as the prophase-triggering Cdk1·cyclin B1 would lethally trigger premature prophase.

A common reason for this attempt to force neurons to suicidally start building up to DNA replication by the piecemeal expression of CDKs and their cyclin components is likely to be the CDK-induced inactivation of the Rb pocket protein and the inappropriate expression E2F1, which, as we learned earlier, is the one member of the E2F family that can induce a $p19^{ARF}$-driven stabilization and accumulation of p53(TP53). Without the checks, balances, and protector proteins of a complete or balanced $G_1$ buildup, the accumulating p53(TP53) stimulates the expression of the $p21^{Cip1/WAF1}$ CKI and other apoptogenic genes.[47–50] Indeed, the accumulation of p53(TP53) could explain the observed expression of $p21^{Cip1/WAF1}$ in Alzheimer brains.[155]

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# 2

# Calcium — A Cell Cycle Driver

## The cycle-driving $Ca^{2+}$ signals: a quick preview

*"Calcium signals are used in a large proportion of regulatory pathways and are thus considered ubiquitous in cell biology, yet they are also selective or specific in the control exerted among myriad functions within a common cytoplasm. How this complex messaging is achieved through a single ion, calcium, remains unsolved."*[1]

Now that we have finished our quick tour of the cell cycle, we will turn around and go back to see where, and maybe how, $Ca^{2+}$ promotes the key cell cycle transitions. Essential though $Ca^{2+}$ and its sensors and effectors, most especially CaM (calmodulin), are for driving cells through their cycle, they have *never* been at the forefront of cell cycle research![1a,2] Indeed, they are rarely, if ever, mentioned in reviews of the CDKs and events such as the initiation of DNA replication and prophase where in fact they play major, if not principal, triggering roles. Thus, the best we can do here is weave what little information we have into a tantalizing tapestry of "educated guesses" of how the ion and its effectors trigger DNA replication, mitosis, and drive cell division (cytokinesis).

The roles of $Ca^{2+}$ transients and oscillations and the corresponding cresting and crashing waves of CaM are just as important as the cresting and crashing waves of CDKs in the $G_0 \rightarrow G_1$, $G_1 \rightarrow S$, $G_2 \rightarrow$prophase, and metaphase→anaphase transitions and the pinching of the cell into its two daughters. So far, we only know that what crudely seem to be identical $Ca^{2+}$ surges or oscillations drive the major parts of the cell cycle. However, shifting patterns of spatially restricted $Ca^{2+}$ sparks, spikes, and waves ($Ca^{2+}$'s diffusion radius is only about 0.1 μm) will light up different cellular regions and targets as the cell progresses through the different stages of the cycle.[3–5] Indeed, mapping the four-dimensional topography of $Ca^{2+}$ signals during the cell cycle is the first prerequisite for understanding the ion's grasp on the cell cycle controls. Only now are we on the verge of being able to do this.[1–2]

## $Ca^{2+}$ and starting the cycle

We start with a snoozing cell as we did in the last chapter. It may be a cultured cell that has lapsed into reversible, $G_0$ quiescence because of a lack of serum growth factors and will start a cycle upon the appearance of the appropriate growth factor(s), or it may be a hepatocyte in a normal adult liver working hard to maintain essential body-wide functions while starting a cycle. Or it may be a small, minimally functioning small lymphocyte with a thin rim of cytoplasm wrapped around a small nucleus with highly

condensed, relatively inactive masses of chromatin, making and then wasting ribosomal RNA. We must note that quiescent, $G_0$ cultured cells, unlike the differentiated cells, are ready to respond to a mitogen by keeping a pool of untranslated messages from cycle-starter genes. This is why the so-called "delayed–early" genes in regenerating liver are the "immediate–early" genes in cultured liver cells of established lines.[6]

The quiescent cell has triggered its cycle-shutdown program. It has planted in its membrane the Gas-1 protein that somehow contributes to the suppression of proliferation.[7–9] It also has installed in its nuclear membrane the 56-kDa phosphoprotein statin complexed with a 45-kDa serine/threonine protein kinase.[10–16] It has upregulated the expression of $p27^{Kip1}$, which would block any low-level surge of Cdk4(or 6)·cyclin D activity.

The silencing of growth factor receptors has affected all aspects of RNA and protein metabolism as well as the mitochondrial energy supply. One key to how a cell switches in and out of the cycling mode is the low priority for access to the translation machinery of the messages from genes encoding growth-regulating transactivators and other regulators. The low position of these transcripts on the translation totem pole is due to their having extensive secondary structure in "capped," 5′-upstream untranslated regions, which have to be "melted" by growth-factor-activated translation initiation factors belonging to the eIF-4 family before they can bind to 43-S pre-initiation complexes and be translated.[17–35] The shutdown mechanism stops the processing of the proliferation-driving gene transcripts, demobilizes the "weak" messages for growth-promoting peptides, reduces the total mRNA content, decreases ribosomal RNA synthesis, increases ribosomal RNA degradation, increases protein degradation by lysosomes, reduces the flow into the nucleus of short-lived non-histone proteins such as transcription factors, and lowers mitochondrial activity.[36] Only the "strong" messages (with their low 5′-secondary structure) for basic housekeeping proteins, proteins for specific tissue functions, and quiescence-maintaining proteins are translated while the number of polyribosomes drops dramatically as the cell shunts much of its "weak" mRNA into a non-polysomal pool.[36] The drop in the translatability of the "weak" transcripts is due mainly to two things. When growth-factor-activated receptor protein kinases fall silent, components of the eIF-4 factor, which limit the translation rate, are dephosphorylated and inactivated.[17–35] Thus, there is a lack of $phosphoSer^{209}$-eIF-4E in the eIF-4 complexes to bind to the 5′-$m^7$GpppX caps of the growth-regulated transcripts and to let the 50-kDa eIF-4A helicase component of eIF-4 complexes melt the transcripts' excessively structured 5′-untranslated regions to enable them to bind to eIF-2·GTP·Met-tRNAf·40-S ribosome pre-initiation complexes.[17–35] The silencing of the growth factor receptors, and consequently the Rsk Ser/Thr protein kinases, results in the dephosphorylation and inactivation of the 40-S ribosome S6 protein components that are needed to form the eIF-2·GTP·Met-tRNAf·40-S pre-initiation complexes.[24]

A signal to a quiescent, cultured cell from a growth factor such as PDGF (platelet-derived growth factor), to a hepatocyte in the adult rat liver from an endogenous growth factor such as the hepatocyte growth factor released in response to partial hepatectomy, or to a small T-lymphocyte from an macrophage-proffered antigen or a plant lectin, starts the cells growing to a state of competence in which they can respond to a second signal from receptors for an endogenous autocrine (self-produced, self-stimulating) or an exogenous paracrine (neighboring cells-stimulating) factor to start the final run-up to DNA

replication. This first "wake-up" signal triggers a veritable explosion of events. The signal-aroused cell will need much energy, which, as we learned in the previous chapter, it gets from glucose, the uptake of which is enhanced by c-Myc. The energy is supplied in the form of ATP by mitochondria lying against the $Ca^{2+}$-loaded endoplasmic reticulum.[36a] This snuggling of mitochondria up to the endoplasmic reticulum is a clever way to ensure that signal-triggered activities will be adequately fueled. The signals from the receptors target the endoplasmic reticulum and cause it to dump a large amount of $Ca^{2+}$ onto the adjacent mitochondria, which increases its ATP production when it picks up this $Ca^{2+}$.[36a,37] The surging $Ca^{2+}$ also activates the cytoskeleton, a striking effect of which is cell surface ruffling, and the nuclear actomyosin motor is switched on to reposition the chromatin to receive and respond to incoming transcription factors and protein kinase and phosphatase modulators of nuclear transcription factors already attached to their target genes' promoters.[38,39] As we learned in the last chapter, at the heart of the cascade are the c-Myc·Max complexes that stimulate the expression of the genes for BN51, eIF-4E, nucleolin, ODC (ornithine decarboxylase) and through their products the mobilization and (or) translation of the transcripts for cycle-starters such as CAK, Cdk4, cyclin D2, c-Fos, c-Fra, ODC, and those of c-Myc itself.[36] To make these and all of the other proteins needed for growth, the cell stops degrading ribosomal RNA and lets it accumulate. Lysosomal degradation stops and a large number of housekeeping proteins and cycle drivers, such as Cdk4 and cyclin Ds to make the cycle-starting CDK, Cdk·cyclin D and CAK to activate it; transcription factors such as c-Fos, c-Myc, NFAT (nuclear factor of activated T-lymphocytes), NF-κB, Stat3; and non-histone chromatin-decondensing proteins are rapidly shipped to the nucleus to reconfigure the chromatin, stimulate the first wave of proliferogene expression, and start the cycle-driving parade of CDKs.[6,36] Putrescine-ODC subunits of RNA polymerase I, addressed specifically to the nucleolus and along with nucleolin, are shipped into the nucleus to stimulate ribosomal RNA production.[36]

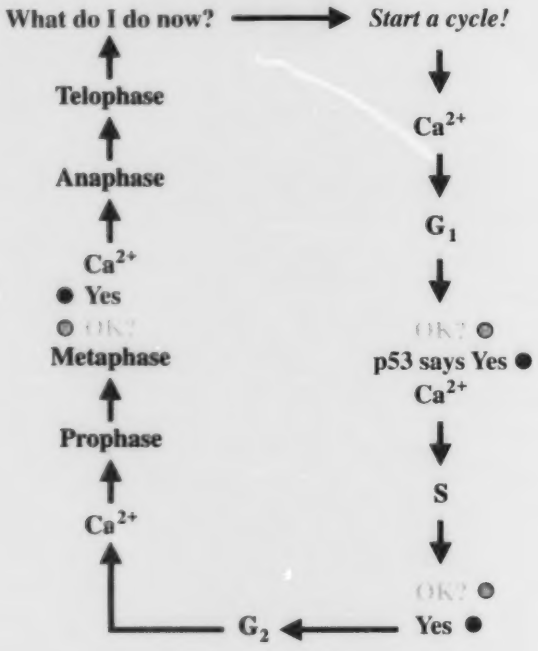
What gives this wake-up call and how does it do it? Among the most common cellular alarm clocks are the growth factor "velcroceptors." Ligand binding to the extracellular parts of the monomeric velcroceptors (e.g., EGF, PDGF), heterodimeric velcroceptors (e.g., IGF-I, insulin), or multi-component or modular velcroceptors (e.g., the T-cell receptor, or the IL-6 receptor with its associate JAK protein tyrosine kinase) causes the receptor molecules to activate their cytoplasmic PYK (protein tyrosine kinase) domains, and cross-phosphorylate each other's tyrosine residues.[40,41] Of course, the monomeric receptors and modular receptors must dimerize and (or) collaborate with PYKs to get their tyrosine residues phosphorylated, while the heterodimeric receptors are already configured for cross-phosphorylation. This *velcroizes* the receptor, which can then hook onto a variety of co-signalers by shoving its phosphorylated tyrosines into the co-signalers' SH2 (Sarc Homologous 2) pockets where the tyrosines' phosphates are grabbed and held by the terminal guanido groups of an arginine residue.[42–47] Velcroization is a wonderful way of luring a team of signal enzymes and adaptors to a place where they can be coordinately phosphorylated by the receptor dimers' protein tyrosine kinase domains, and interact with each other and other membrane-associated molecules to switch on several differently targeted signal mechanisms. In other words, velcroization enables a single receptor to coordinately activate a specific panel of signal mediators, the velcrocluster, and thus simultaneously turn on several different signal

mechanisms.[41–47] The kinds of SH2-bearing co-signalers in a velcrocluster or velcroceptor component depends on the amino acids flanking the velcroceptor's phosphotyrosine residues and the co-signaler's SH2 pockets.[41–47] For example, the phospho-$Tyr^{739}$ residue of the β-PDGF receptor, with its associated phospho-Tyr–Asn–Ala–Phe–Tyr sequence, grabs Ras-GAP (GTPase-activating protein); the receptor's phospho-$Tyr^{1021}$, with its associated phospho-Tyr–Ile–ile–Phe–Tyr sequence, hooks phospholipase-$C\gamma_1$; phospho-$Tyr^{708}$ and phospho-$Tyr^{719}$, with their associated phospho-Tyr–Met–Asp–Met–Ser and phospho-Tyr–Val–Phe–Met–Leu sequences, grab the 85-kDa regulatory subunit of PtdIns-3K; and the phospho-$Tyr^{579}$ residue, with its associated phospho-Tyr–Cys–Val–Asp sequence, hooks Src-family protein tyrosine kinases.[36] Thus, differently sited phospho-velcroceptors, and the different adaptors, such as IRS1 and Shc, that they phosphorylate, recruit different sets of signalers, which in turn trigger different signal cascades in different parts of the cell.[41,48,49]

To awaken a quiescent cell, we may induce it to express the first set of proliferogenes by activating appropriate mitogenic velcroceptors on its surface. The signals from such receptors will activate the transcription factors that will turn these genes on. In this type of activation, things start when Grb2 adaptors shove the receptor's appropriately identified phospho-Tyr residues into their SH2 pockets, which causes the adaptor to expose its SH3 motif.[41,50] It then binds and thereby activates mSos, which, in turn, activates membrane-bound Ras molecules by stimulating them to replace their attached GDP with GTP. The now activated GTP·Ras interacts with and activates the 74-kDa Raf-1 Ser/Thr protein kinase.[36,41,48–51] The activated Raf-1 kinase then phosphorylates and activates an enzyme known as MEK, which is a dual-function Ser/Thr/Tyr-protein kinase.[41,52] MEK then activates the Ser/Thr MAP kinase (MAPK) by phosphorylating the enzyme's $Thr^{183}$ and $Tyr^{185}$ residues.[52,53] The activated MAP kinase can enter the nucleus to phosphorylate and thus activate a variety of transcription factors such as the multipurpose c-Jun and the exclusively proliferation-related c-Myc whose targets are key proliferogenes such as the Cdk4·cyclin D-activating CAK.[54–59] Another key target of the MAP kinases are the S6 Ser/Thr protein kinases, which are activated and phosphorylate five serine residues of the S6 subunits of 40-S ribosomes.[36] These five residues are on the "platform" of the 40-S ribosome, which is involved in codon:anticodon tRNA binding. This phosphorylation triggers a configurational change, which increases the 40-S subunit's message-binding ability.[24] Yet, another result of this cascade is the phosphorylation and activation of eIF-4E's $Ser^{209}$, which enables the protein to form complexes with the eIF-4A helicase that mobilize growth-driving messages by melting the extensive secondary structure of their capped 5′-untranslated regions and binding them to the primed, S6-phosphorylated, 43-S pre-initiation complexes.[32–36] All of this results in a wave of protein synthesis, particularly the synthesis of the key proteins that will eventually start the parade of cycle-driving cyclins and cyclin-dependent protein kinases.

However, I have not mentioned the hero of this chapter and book, $Ca^{2+}$. Indeed, velcroceptor-started cycles (there are other starters as we will see further on) start with a $Ca^{2+}$ spike or oscillations (Fig. 2.1). The $Ca^{2+}$ story starts with the auto(Tyr) phosphorylated velcroized receptor, grabbing phospholipase-Cγ by its SH2 pockets. The activated phospholipase stimulates the breakdown of PtdIns(4,5)$P_2$ (phosphatidylinositol-4,5-bisphosphate) into Ins(1,4,5)$P_3$ (inositol-1,4,5-trisphosphate) and a first wave of short-lived diacylglycerols.[36]

**Fig. 2.1.** The $Ca^{2+}$ signals that have been found to trigger the major stages of the cell cycle.[36,71,196] A $Ca^{2+}$ surge is involved in starting the $G_1$ buildup. In some cells, this $Ca^{2+}$ comes mainly from internal stores, while in other cells an essential part of the $Ca^{2+}$comes through membrane channels. Therefore, external $Ca^{2+}$ deprivation prevents cells with inadequate internal $Ca^{2+}$ stores from starting the $G_1$ buildup, while it stops other cells starting out with adequate stores only when they near end of the buildup where $Ca^{2+}$ oscillations are involved in triggering chromosome replication. Then come the $Ca^{2+}$ surges that trigger prophase and later anaphase. This figure is actually just Fig. 1.1 with both the checkpoints (◎●) and $Ca^{2+}$ signaling points indicated.




First, let us look at the diacylglycerols. They stimulate nine (α, βI, βII, γ, δ, η, ϵ, θ, μ) of a family of 12 Ser/Thr protein kinases, the PKCs (protein kinase-Cs).[60,61] At low concentrations, the diacylglycerols stimulate any inactive PKCs that the cell might be harboring in its plasma membrane, but at higher concentrations they can drive more PKCs from the cytosol onto activation sites in the plasma membrane with the help of an Ins(1,4,5)$P_3$ -induced release of $Ca^{2+}$ from internal stores.[62] The membrane-associated PKCs phosphorylate and inhibit some receptors such as the EGF/TGF-α receptors and enzymes such as the phospholipase-Cγ that generated them and then phosphorylate and modulate the activities of a host of other enzymes, signal mechanisms, and gene transcription factors around which a huge literature has collected and will not be discussed until the next chapters.[36,62] The first wave of diacylglycerols crashes within a few seconds as the membrane-associated PKCs phosphorylate and silence phospholipase-Cγ.[36] However, while they have shut down phospholipase-Cγ, they have stimulated another phospholipase, phospholipase-D, which breaks phosphatidyl choline down into phosphatidic acid and choline.[36] The phosphatidic acid is then dephosphorylated to

diacylglycerol, which re-activates PKCs, thus setting up a positive-feedback stimulation that produces a second, but now sustained, surge of membrane-associated PKCs activity.[36]

The transient surge of Ins(1,4,5)$P_3$ that accompanies the brief burst of phospholipase-Cγ activity does a very important thing. It binds to 260-kDa Ins(1,4,5)$P_3$ receptors/channels on $Ca^{2+}$- storage vesicles, which may bud from, or remain as specialized regions of, the endoplasmic reticulum.[63–68] The emptying of the $Ca^{2+}$ stores triggers, via a message from the Ins(1,4,5)$P_3$ receptors' heads to the cell membrane, the opening of CRAC ($Ca^{2+}$-release-activated) or SOC (stores-operated) channels.[63,65,69] The upshot of this is the transient burst of intracellular $Ca^{2+}$ oscillations that marks the start of the G1 buildup in a wide variety of cells.[36,63,70,71] The Ins(1,4,5)$P_3$ generated at the velcroceptor sites in the cell membrane spreads to the underlying nucleus and triggers the release of $Ca^{2+}$ from the nuclear envelope stores into the nucleus through Ins(1,4,5)$P_3$ receptor/channels.[72–76] The surging $Ca^{2+}$ forms complexes with a variety of cytoskeletal and other proteins, perhaps the most important of which are the complexes with CaM (calmodulin), $Ca^{2+}$·CaM. The $Ca^{2+}$ surge also facilitates the movement of intermediately-sized (around 10 kDa) molecules into the nucleus by removing a central plug or gate in the nuclear membrane pores.[77,78]

The surging $Ca^{2+}$ and with it a wave of $Ca^{2+}$·CaM complexes activate a formidable array of enzymes such as calcineurin (a protein Ser/Thr phosphatase), CaMKII (calmodulin-dependent Ser/Thr protein kinase), CaMKIV, and MLCK (myosin light-chain kinase). The nuclear $Ca^{2+}$ content shoots upwards and the resulting $Ca^{2+}$·CaM complexes do two important things. They activate MLCK and thus stimulate the nuclear actomyosin machinery to move the chromatin into a responsive configuration.[38,39] And they stimulate the expression in the quiescent cell of at least one key proliferogene, c-*myc*, by binding to and displacing the transcriptionally inactive Max/Max homodimers from the gene's promoter, which enables their replacement by transcriptionally active Myc/Max heterodimers.[58,79] Some cells, such as corticotrophs, lymphocytes, neurons (which can respond to proliferogenic signals by growing processes, but generally cannot replicate their possibly unlicensed chromosomes [see previous chapter]), and reproductive cells, have CaMKIV, which, unlike most CaMKII isoforms (although δ-CaMKII does have a nuclear localization motif), can enter the nucleus where, among other things, it can mimic cyclic AMP by activating an "immediate–early" cyclic AMP-responsive gene such as c-*fos* by phosphorylating and activating the CREB (cyclic AMP-responsive element [CRE]-binding protein) protein sitting on the CaRE ($Ca^{2+}$-responsive element with a consensus CRE motif) element of its promoter in response to a nuclear $Ca^{2+}$ surge.[80–82] In another example, the $Ca^{2+}$ surge triggered by the modular T-cell velcroceptor activates the calcineurin phosphatase, which associates with and dephosphorylates the cytoplasmic NFAT1 (Nuclear Factor of Activated T-cells), which then translocates to the nucleus where it attaches to five sites in the promoter of the IL(interleukin)-2 gene, at four of which it joins with the AP-1 (c-Fos/c-Jun) transactivation complex.[83–89] The result of this is the production of IL-2, which triggers the second stage of the growing T-cell's $G_1$ buildup.[36]

We learned in the previous chapter that Myc·Max transactivates the genes for two very important things needed to turn on Cdk4(or 6)·cyclin D, the $G_1$-starting CDK. First are the components of Cdk7·cyclin H, CAK, which besides being involved in activating

Cdk4(or 6)·cyclin D, is part of the RNA polymerase II transcriber's pre-initiation complex when associated with the MAT1(p36) protein.[57] Second is the early $G_1$-specific, CDKs-activating STY phosphatase Cdc25A, which like its $G_2$-specific isoform, Cdc25C, might be activated by being phosphorylated by $Ca^{2+}$·CaM-stimulated CaMKII.[90] Indeed, specifically blocking CaMKII activity with KN-93 greatly reduces the level of cyclin D1 and blocks the $G_1$ buildup in NIH3T3 mouse cells.[70–91]

Starting a cycle can be lethal for a normal cell if there is only an incomplete, thus "unbalanced," $G_1$ buildup.[92–99] Thus, for example, hyperexpressing the c-*myc* proliferation-starter gene in normal fibroblasts without the support of protector signals from serum factors indeed leads to the overexpression of E2F1, which stimulates the accumulation of the apoptogenic p53 and also the expression of cycle-driving components such as cyclin A and Cdk1·cyclin B1, which are apoptogenic when expressed out of context and without protectors such as Bcl-2 and the anti-p53 Mdm2.[54–56,92,93,96,97] The signal must trigger *in the proper order* the first wave of cyclins, the cyclin Ds, and the assembly of active Cdk4·cyclin D protein kinases that start the $G_1$ buildup (Fig. 1.2). However, it must also supply the protectors and downregulate the promiscuous CDK inhibitor $p21^{Cip1/WAF1}$ that is stimulated to increase by the mitogenic signal.[92–99] To do this it must receive converging streams of signals from serum factors and a subset of β1 integrin receptors that are activated by binding to substrate proteins such as fibronectin and vitronectin.[92–99] As we learned in the previous chapter and will continue to learn in the next chapters, the integrin signals provide the MAPK, PtdIns-3K, and ILK activities that stimulate the expression of the Bcl-2 protector, suppress the apoptogenic BAD protein, suppress the apoptogenic caspase proteases, and snatch β-catenin from the jaws of the proteasome shredder so that it can get into the nucleus to stimulate its several proliferogenic target genes. Therefore, detaching various normal activated cells from culture vessel or their appropriate basal lamina triggers the apoptogenic mechanism, a response that we learned in the previous chapter is called *anoikis* (from the Greek word for homelessness).[98] This suicide is extremely important for a multicellular society where a proliferatively competent, unattached cell and its progeny would wreak social havoc, but in the next chapter we will see how anoikis has been co-opted for the *diffpoptosis* (differentiation-apoptosis) of keratinocytes. $Ca^{2+}$ is involved in the final mechanism that ultimately kills all apoptosing cells by inducing the collapse of the potential across the inner mitochondrial membrane ($\Delta\psi_m$), which opens Bcl-2-blockable permeability transition pores (megachannels) through which a 50-kDa caspase (formerly known as ICE[Interleukin-1β-like Converting Enzyme]-like cysteine protease) moves from the mitochondrion into the cytoplasm and triggers the apoptogenic cascade of protease activities.[98,100–107] How can this happen? Why must the cell activate a parallel survival mechanism to protect itself from the mitogenic signal? The answer may lie in the $Ca^{2+}$ oscillations and oxidizing radicals triggered by the burst of velcroceptor signaling.[36,108,109] This causes the mitochondria to accumulate $Ca^{2+}$,[108] which can convert the membrane's ADP/ATP transporter into a large channel (i.e., the channel has a very large conductivity for cations of 300–600 pSiemens)[110–112], which, if not controlled by Bcl-2 and (or) other factors enhanced by the survival arm of the cycle-driving mechanism, will trigger the irreversible common caspase-mediated execution phase of apoptosis. (Incidentally, megachannel formation in the inner mitochondrial membrane is enhanced by inorganic phosphate,[113] which explains Whitfield's 36-year-old observation that radiation-induced

apoptosis in thymic lymphocytes is potently primed by inorganic phosphate,[36,114] and Meleti et al.'s much more recent observation that inorganic phosphate induces apoptosis in cultured human osteoblasts.[114a])

However, there is a different cycle-starting pathway, the specialized cyclic AMP/PKA (cyclic AMP-dependent protein kinase) pathway, which, like the velcroceptors' MAPKs/PKCs default pathway, converges at the surge of Cdk4·cyclin D activity needed to liberate E2F from the grip of the Rb-family pocket proteins and starts the waves of gene expressions and CDKs, leading to chromosome replication.[36,115–118] The cyclic AMP/PKA pathway is used by many types of cell, including many, if not most, differentiated epithelial secretory cells such as adrenocortical cells, parotid gland acinar cells, pancreatic cells, pituitary somatotrophs, and thyrocytes.[36,115–118] Such cells have a special mitogenic mechanism that allows them to replace lost functioning cells or to respond to an increased functional demand by triggering a transient burst of proliferation, which is followed by the prompt withdrawal of the new cells into the functional pool.[36,115–118]

The cyclic AMP/PKA mechanism has been most intensely studied in dog thyroid cells stimulated to proliferate by TSH (thyroid-stimulating hormone).[116,118] Adding an optimally effective dose of TSH to a primary culture of dog thyrocytes triggers a 48-h surge of cyclic AMP and proliferation-specific PKA activity (TSH's receptor is a slowly downregulated member of the serpentine or 7-TMD [transmembrane domain] family of G-protein-activating receptors) with none of the protein tyrosine kinase activation, phospholipase-Cγ activation, PtdIns(4,5)$P_2$ breakdown, surge of PKCs activities on the plasma membrane or in the nucleus, or $Ca^{2+}$ transient that would be triggered in the same cells by EGF or hepatocyte growth factor velcroceptors.[116–120] Like the signals from velcroceptors, the cyclic AMP/PKA signal from TSH receptors downregulate the expression of a statin-related gene and stimulate the expression of the c-*myc* gene, but unlike this gene's prolonged elevation in response to signals from mitogenic velcroceptors, the c-*myc* response to the mitogenic cyclic AMP signal peaks at 1 h only to crash by 3 h.[116,118,121] Cyclic AMP/PKA initially lifts a transcriptional elongation block, which is followed 30 min later by the stabilization of the mRNA transcripts.[121] In a second stage, and in stark contrast to the sustained c-*myc* expression in velcroceptor-stimulated cells, the c-*myc* transcripts are destabilized and transcription is again shut off by the appearance of a protein that limits the mitogenic response to allow the resumption of function.[116,118,121] The mitogenic, $Ca^{2+}$-mobilizing velcroceptor signals strongly stimulate both c-*jun* and *jun D* gene expression, while the cyclic AMP/PKA signal inhibits c-*jun* and stimulates *jun D* expression.[116] Since cyclic AMP/PKA actually inhibits Raf-1; there is *no* Ras/Raf 1-mediated MAP kinase cascade in the cyclic AMP/PKA-stimulated thyrocytes as there is in velcroceptor-stimulated thyrocytes; there is no phosphorylation/activation of c-Jun proteins by activated phospho-MAP kinases surging into the nuclei; and because of this plus the failure to increase c-Jun production, there is none of the high, differentiation-suppressing AP1 activity that characterizes velcroceptor-stimulated cells.[116]

The cyclic AMP/PKA signal starts the cells growing and expressing and accumulating the RIα PKA regulatory subunits to make the PKAI isozymes needed to drive the $G_1$ buildup and sets the cell on a path that must include the Cdk4/6·cyclin D-triggered cresting and crashing waves of CDKs' activities.[116,122–125] However, cyclic AMP/PKA do

not stimulate, and may even reduce, the expression of the cyclin Ds![126] But there is a very big problem with this! How do they stimulate proliferation when, as we learned in the previous chapter, activated Cdk4/6·cyclin Ds must accumulate above a critical level in the nucleus to inactivate the pocket proteins and release E2F to stimulate the cycle-starting genes? The answer is delightfully simple. Cyclic AMP/PKA enhances the assembly of Cdk4·cyclin D3 by increasing cyclin D3's affinity for Cdk4 and stimulates the transport of the Cdk4·cyclin D3s into the nucleus.[126]

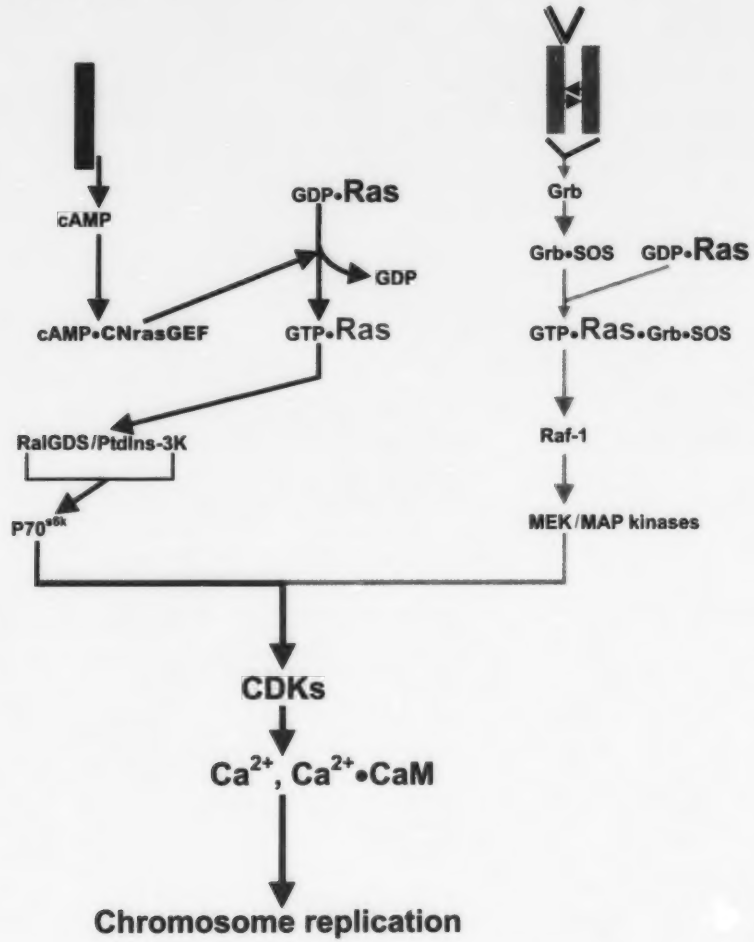
These cyclic AMP/PKA-stimulated cells, just like cells stimulated by a mitogenic velcroceptor, now briefly need second signals from a velcroceptor to stimulate growth and the accumulation of these and other components such as Cdk2·cyclin E and Cdk2·cyclin A to their critical threshold levels and the $Ca^{2+}$ surge needed to drive the cells into the S phase.[116,122–125] In velcroceptor-activated thyrocytes, this second velcroceptor signal is from insulin or IGF-I receptors, but in the cyclic AMP/PKA-activated thyrocytes the second signal is from insulin velcroceptors that have been accumulating during the $G_1$ buildup.[127]

But how do cyclic AMP and the PKAs start the drive to DNA replication and mitosis? Cultured dog thyroid cells, Schwann cells, and Swiss albino 3T3 mouse cells have both the cyclic AMP/PKA proliferogenic response mechanism and the PtdIns(4,5)$P_2$/Ras/Raf-1/MEK/MAP default proliferogenic mechanism. Other cells such as NIH3T3 cells have only the default mechanism: they cannot be stimulated to proliferate by cyclic AMP/PKA.[128] In the cyclic AMP-responsive cells, the cyclic nucleotide-triggered mechanism bypasses the head of the default mechanism to start the $G_1$ buildup by activating Ras, which in turn stimulates its effectors RalGDS and PtdIns-3K (phosphoinositide-D3 kinase), which phosphorylates and stimulates $p70^{s6k}$ after $p70^{s6k}$ has relocated to the cell membrane.[128–133] The activated $pp70^{s6k}$ protein kinase then enters the nucleus and activates the expression of key cycle-starting genes by phosphorylating CREM (cyclic AMP-response element modulator) transactivators on cyclic AMP-response elements in their promoters.[134] This protein kinase has the added feature of enhancing protein synthesis by phosphorylating the target for which it was named, the ribosomal S6 subunit discussed above.

So far we have assumed that the TSH-stimulated cyclic AMP surge in a thyrocyte starts the buildup to chromosome replication by only activating PKAs. Nevertheless, it now looks as if things are not so "simple." While a cyclic AMP surge stimulates thyrocyte proliferation, microinjecting active PKA catalytic subunits does not.[134a] It seems likely that cyclic AMP operates in part by binding directly to a protein, CNrasGEF (Cyclic Nucleotide ras Guanine nucleotide Exchange Factor), that activates Ras by stimulating the exchange of the GDP in inactive GDP·Ras for GTP to produce active GTP·Ras complexes (Fig. 2.2).[134b] The activated GTP·Ras complexes then operate through RalGDS and PtdIns-3K to activate $p70^{s6k}$, which bypasses the Raf-1/MAPK default mechanism (Fig. 2.2) and collaborates with equally essential PKA-stimulated processes to start the buildup to chromosome replication. These recent observations suggest that expressing CNrasGEF is why some cells but not others can be stimulated to proliferate by things that cause cyclic AMP surges.

During the last quarter century, it has become clear that $Ca^{2+}$ released from internal stores with or without $Ca^{2+}$ flowing in through opened CRAC (SOC) channels is necessary to start the cycles of a variety of cells with velcroceptor-activating mitogens[36,70] but,

**Fig. 2.2.** Why can cyclic AMP stimulate some cells such as thyrocytes to start building up to chromosome replication when it (via the PKAs it activates) interferes with growth-factor velcroceptors (V) signals from activating the MEK/MAP kinase mechanism that starts the buildup in many other types of cell? A possible answer resides in the ability of some cells to express CNrasGEF, which directly binds cyclic AMP and then activates Ras by stimulating the exchange of GDP for GTP as shown in the drawing. The activated GTP·Ras then uses the alternative or bypass mechanism shown here to switch on the required final common mechanism involving the CDKs, the stimulation of replication-related genes, and the various actions of $Ca^{2+}$ and $Ca^{2+}$·CaM.




as we have just seen, apparently *not* in epithelial cells that have been additionally equipped with an activated cyclic AMP/PKA mitogenic mechanism. The velcroceptor/PLC/$Ca^{2+}$/PKC mechanism is the default mechanism that suppresses differentiation and promotes sustained cycling, while the cyclic AMP/PKA mechanism is designed for limited cycling and maintenance of differentiated functions. However, as discussed in the previous chapter, there is a seemingly universal later G1 cyclic AMP surge in

normal cells and some cancer cells which has been proven to be needed to complete the buildup to DNA replication.[36,115] But it should be kept in mind that there is no distinct later $G_1$ surge in TSH -stimulated thyrocytes because of the prolonged elevation of the cyclic AMP level by the slowly deactivating TSH receptor, although there is a small, second cyclic AMP surge in isoproterenol-activated parotid gland acinar cells which is necessary because of the rapid deactivation of the adenylyl cyclase-activating, cyclic AMP-elevating β-adrenergic receptors.[36] This later $G_1$ surge is controlled by external $Ca^{2+}$ in the hepatocytes of primary neonatal rat liver cultures. These cells, just like hepatocytes activated by partial hepatectomy in the adult rat liver, generate both an early and the later $G_1$ cyclic AMP surge when stimulated by EGF in serum-free, synthetic medium containing 1.0 mM $Ca^{2+}$, but while they still make the same amounts of cyclic AMP when stimulated by EGF in medium containing only 0.02 mM $Ca^{2+}$, they dump the cyclic nucleotide into the medium and thus cannot generate the much-needed later internal $G_1$ surge.[135] Thus, external $Ca^{2+}$ and therefore maybe signals from the hepatocyte's surface $Ca^{2+}$ sensor/receptor, which we will formally meet further on, restrain the activity of the transporter-mediated mechanism that drives cyclic AMP efflux.[135a] However, while such a severe external $Ca^{2+}$ deprivation is attainable in cell cultures, it is not attainable in a rat; the blood $Ca^{2+}$ concentration does not fall below 0.5 mM after removal of the parathyroid glands. In the rat, the relatively small (50%) reduction of the plasma $Ca^{2+}$ concentration and the resulting severe drop in plasma $1\alpha,25(OH)_2$ vitamin $D_3$ concentration that develop by 3 days after parathyroid removal do *not* prevent the later $G_1$ cyclic AMP surge in hepatocytes after their proliferative activation by partial hepatectomy, but they prevent the surge of the PKAs' common catalytic subunit without affecting the surge of their RI subunits and thus contribute to the failure of the hepatocytes to initiate DNA replication.[36] As we learned in the previous chapter, the responses to this failure are likely to be failures to stimulate cyclin A expression, inadequate delivery of cyclin A and other key components into the nucleus, and consequently an inability to initiate DNA replication.

## $Ca^{2+}$ and the $G_1 \rightarrow S$ transition

As the cell, be it a hepatocyte in the regenerating rat liver, a T51B rat liver cell, a phytohemagglutinin-activated T-lymphocyte, a NRK kidney-derived rat cell, or a thyrocyte, advances to the Cdk2·cyclin E- and the Cdk2·cyclin A-driven stages of the $G_1$ buildup, it starts expressing one or more of its three non-allelic calmodulin genes with identical (80–85%) coding regions but different non-coding regions that produce an identical product.[135b] The cell accumulates this key sensor/signaler in preparation for the $Ca^{2+}$-dependent triggering of DNA replication.[36,136–143] As the normal cell nears the end of the $G_1$ buildup, it becomes extremely sensitive to external $Ca^{2+}$ deprivation. For example, hepatocytes activated by partial hepatectomy in a rat 24 h after it has had its plasma $Ca^{2+}$ concentration halved or nearly halved by thyroparathyroidectomy cannot accumulate ribonucleotide reductase subunits and thymidylate synthase and thus cannot make the deoxyribonucleoside triphosphates needed to start replicating DNA, but injecting $CaCl_2$ (or PTH — hepatocytes express PTH/PTHrP receptors[144]) intraperitoneally to produce a transient pulse of plasma $Ca^{2+}$ between 12 and 15 h after the partial hepatectomy enables the cells to start replicating DNA on time between 18 and 20 h; but a later $Ca^{2+}$ injection does not work: a window of opportunity has been closed.[36,145]

For the last two decades, it seemed that normal hepatocytes in the liver of a rat could sense and respond like parathyroid gland chief cells to changes in the extracellular $Ca^{2+}$ concentration. Now we know that hepatocytes do indeed have the same low-affinity CaRs ($Ca^{2+}$or divalent cation receptors) (high-affinity receptors would be locked in the active state by even the lowest possible blood $Ca^{2+}$ concentrations) as the parathyroid cells and, as we shall see in the next two chapters, keratinocytes and colon cells.[146-147] Evidently, cells in various tissues of the body need to be able to sense the external $Ca^{2+}$ concentration. Thus, it seems at least worth considering the possibility that an increasing volume of signals from CaRs accumulating on the plasma membrane during the $G_1$ buildup might stimulate PLC-$\gamma$1 to produce an Ins(1,4,5)$P_3$-induced release of $Ca^{2+}$ from internal stores and diacylglycerol-stimulated membrane-associated PKCs activity, which are needed at a critical point in the $G_1$ buildup. Consequently, halving the extracellular $Ca^{2+}$ concentration by removing the parathyroid glands would stop the buildup by preventing the CaR signaling from reaching a critical level.

$Ca^{2+}$ injection (or a *single* injection of PTH) by itself does not enable the hepatocytes in prolonged (e.g., for 72 or 96 h) hypocalcemic TPTX (thyroparathyroidectomized) rats to complete the construction of replication factories and start replicating DNA because of the severe shortage of 1$\alpha$,$(OH)_2$ vitamin $D_3$ that develops after TPTX, and the consequent failure to accumulate enough PKA catalytic subunits and CaRs if 1$\alpha$,$(OH)_2$ vitamin $D_3$ stimulates the expression of the CaR gene in hepatocytes as it does in keratinocytes.[36,146-149] However, when both the blood $Ca^{2+}$ and 1$\alpha$,$(OH)_2$ vitamin $D_3$ concentrations are kept from falling after TPTX by feeding the rats a low-phosphorus diet, hepatocytes can replicate their DNA at the normal time even when they are stimulated by partial hepatectomy at 72 h after TPTX.[150]

Lowering the external $Ca^{2+}$ concentration to a sub-optimal level also stalls BALB/c 3T3 mouse cells, C3H10T1/2 mouse fibroblasts, primary rat thigh muscle cells, T51B rat liver cells, and human WI38 fibroblasts late in the $G_1$ buildup.[36,70] This $Ca^{2+}$-sensitive period is extremely short in Swiss albino 3T3 cells.[151] $Ca^{2+}$ deprivation does not stop these cells from starting a cycle, probably because they, like some other cells driven by the velcroceptor-activated default pathway, release enough $Ca^{2+}$ from their internal stores without needing to depend on $Ca^{2+}$ flowing in from stores-operated CRAC channels.[70,152] (But it does stop cells from starting the $G_1$ buildup if they do not have large enough internal stores.[70,152]) Then, 5-min exposures to $Ca^{2+}$ deprivation prevent the initiation of DNA replication only when carried out within a couple of hours before the expected onset of replication, but is totally ineffective *before* or *after*.[151] In other words, some critical thing requiring external $Ca^{2+}$ appears briefly, does its job, and then disappears and with it the need for external $Ca^{2+}$. However, if the external $Ca^{2+}$ is removed during this critical time, the cells cannot initiate DNA replication, and re-adding $Ca^{2+}$ does not enable them to complete the buildup and initiate DNA replication; an unstable component(s) has been lost and the cell resets itself into a $G_0$ state.[151] BALB/3T3 cells and T51B rat liver cells in confluent cultures, like the cells in the regenerating rat liver in the hypocalcemic, 24-h post-thyroparathyroidectomized rat, also cannot stay long in a late $G_1$ block imposed by an external $Ca^{2+}$ shortage, and they rapidly become unable to immediately restart and finish the buildup if the external $Ca^{2+}$ level is raised; they must reset themselves and be stimulated to start over again.[36] Yet, another indicator of a $Ca^{2+}$-dependent late $G_1$ mechanism is a subpopulation of lymphoblasts in the rat thymus that

are blocked in late $G_1$ poised to start replicating DNA in less than an hour after the addition of $Ca^{2+}$ or an agent that can trigger a $Ca^{2+}$ surge (as well as surges of cyclic AMP or cyclic AMP-elevators).[36]

After accumulating in the cytosol, CaM is shipped into the nucleus, and the nuclear calmodulin content, for example, in regenerating liver cells, as much as triples.[72] This CaM may stimulate the transport of Cdk4·cyclin D into the nucleus where it can associate with the chromosomes and hyperphosphorylate and disable the gene-suppressing pocket proteins we met in the previous chapter.[153–155] CaM does not simply diffuse through the nuclear pores, although it is small enough to do so.[39] Instead, it binds to $p21^{Cip1/WAF1}$. CDk4·cyclin D complexes, which would expose p21's nuclear localization sequence and use it to trigger the shipment of the CaM·$p21^{Cip1/WAF1}$·CDk4·cyclin D complex into the nucleus.[153]

The nuclear envelope, a specialized extension of the ER (endoplasmic reticulum), has the ER's Ins(1,4,5)$P_3$ receptor/channels, ryanodine receptors (opened by cyclic AMP-ribose), and $Ca^{2+}$ pumps.[36a,39] Something now triggers the late-$G_1$ $Ca^{2+}$ signaling (Fig. 2.1) that in C127 mouse mammary cells starts in the perinuclear region probably upon the emptying of the Ins(1,4,5)$P_3$-responsive stores in the nuclear membrane.[1,72–75,141] Hepatocytes in the regenerating liver prepare for this stage by radically shifting the expression of their $IP_3$Rs (Ins(1,4,5)$P_3$ receptor/channels) from the initially predominant $IP_3$R2s located on apical stores to $IP_3$R1s concentrated on perinuclear stores.[155a] This shift does two things. First, it ensures that $Ca^{2+}$ boluses are aimed directly at nuclear targets. Second, it enables the extra-store $Ca^{2+}$concentration to oscillate because Ins(1,4,5)$P_3$-activated/opened $IP_3$R1s close to enable the stores to refill and thus lower the extra-store $Ca^{2+}$ concentration when the extra-store concentration exceeds a certain level, and then while still activated by Ins(1,4,5)$P_3$ they open again to empty the refilled stores when the extra-store $Ca^{2+}$ concentration falls below the critical level.[155a] Activated $IP_3$R2s do not close when the extra-store $Ca^{2+}$ concentration rises as the stores empty: their activation results simply in a sustained increase in the $Ca^{2+}$concentration.[155a]

Soon the nuclear $IP_3$R1s are activated and the intracellular $Ca^{2+}$ concentration starts oscillating — the cell starts singing its initiating $Ca^{2+}$ song.[71] This signaling depends on an adequate pre-loading of the $Ca^{2+}$ stores by the ER's $Ca^{2+}$ pump (the so-called SERCA pump[36a]). (In regenerating hepatocytes, the SERCA 1 isoform does this job while the cells for some reason downregulate the SERCA 3 isoform.[155a]) Thus, switching off the ER pump's gene delays the onset of DNA replication, which arouses the slumbering $G_1$→S gatekeeper p53(TP53), which increases the expression of the $p21^{Cip1/WAF1}$ cyclin-dependent protein kinase inhibitor.[156] Moreover, emptying the Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ stores (by inhibiting the stores'-refilling ATPase pumps with thapsigargin) of diploid human fibroblasts just before the time when DNA replication should begin prevents the expression of the cyclin A needed to produce the Cdk2·cyclin A that drives DNA replication in the factories.[157]

What drives this final stage of the $G_1$ buildup? From the results of experiments on BALB/c 3T3 cells and vascular smooth muscle cells, it seems that things start with the expression and installation in the cell membrane of $Ca^{2+}$-permeable channels[158] and the late-$G_1$ expression of c- or B-Myb.[159] The c-Myc induced at the start of the buildup by the mitogenic velcroceptor (e.g., the PDGF receptor) stimulates the expression of the IGF-I velcroceptor gene and the subsequent buildup of the receptors to a threshold level at

which, when they are activated by exogenous or autocrine IGF-I, their signals are loud enough to open the newly installed channels in the cell membrane and enable $Ca^{2+}$ to flow into the cell.[158–161] The IGF-I-induced $Ca^{2+}$ influx and the resulting internal $Ca^{2+}$ surge is reinforced by a Myb-induced reduction of $Ca^{2+}$ efflux.[159] However, there is much more than this to the signal from a velcorceptor such as the IGF-I receptor. The Ras-mediated signal from the IGF-I receptors may also selectively target the nuclear envelope and trigger the nuclear PtdIns(4,5)$P_2$ breakdown cascade, as it seems to do in regenerating rat liver cells and Swiss albino 3T3 cells,[162,163] thereby triggering the release of $Ca^{2+}$ from perinuclear stores and the start of the $Ca^{2+}$ oscillations that are needed to start DNA replication. The activated IGF-I receptors also have specific domains that are not involved in the cycle-driving signals, but prevent apoptogenic consequences of the $Ca^{2+}$ oscillations by maintaining the level of the megachannel-blocking Bcl-2 protector protein and preventing a murderous, apoptogenic caspase (ICE-like protease) cascade.[160,164,165] The signals from adhesion-activated β1-integrins and ILK (the integrin-linked protein kinase we will meet in the next chapter) and their targets collaborate with the IGF-I receptors to suppress a caspade (caspase cascade).[99]

We have left the Cdk4·cyclin D part of the buildup and are now in the Cdk2·cyclin E and Cdk·cyclin A stages of the buildup. While the exact relations between the late Cdk2·cyclins, $Ca^{2+}$/PKCs surges, the expression of the replication genes, and the construction of replication factories are far from known, we do have the vague outlines of them. We know that the release of $Ca^{2+}$ from Ins(1,4,5)$P_3$-responsive stores and adhesion-induced signals from the β1-integrin-associated kinases are needed for the expression of cyclin A.[98,99,166–170] We also know that CaM is needed to start replication.

The cell has been loading its nucleus with CaM and the IGF-I/Myb-triggered $Ca^{2+}$ oscillations around the nucleus spawn $Ca^{2+}$·CaM complexes that stimulate the ATPase pumps in the nuclear envelope to pump $Ca^{2+}$ into the nucleus.[72,74] This $Ca^{2+}$ surge also might directly stimulate calpain protease isoforms, which could shred the CKIs that have been restraining the CDKs.[171] Indeed, inhibiting calpain activity stops the $G_1$ buildup in cells such as vascular smooth muscle cells.[1,171] The ion also collaborates with cyclic AMP/PKA[171,172] to provide the open nuclear pores needed at this critical nuclear loading time by binding to the EF-handed, $Ca^{2+}$-binding gp210 pore protein.[39] When it surges into the nucleus, it produces nuclear Ca·CaM complexes and causes CaM to move to the nuclear periphery.[72]

The surging nuclear $Ca^{2+}$·CaM complexes somehow trigger a very late step in the initiation sequence. Thus, DNA replication can be prevented in COS-7 cells by selectively blocking nuclear $Ca^{2+}$·CaM action with an engineered peptide consisting of multiple tandem repeats of the $Ca^{2+}$·CaM-binding domain of myosin light chain kinase and multiple repeats of a nuclear localization sequence to load it into the nucleus.[173] The $Ca^{2+}$·CaM blocker, W-7, a naphthalene sulfonamide, blocks Chinese hamster ovary cells in so late a stage of the $G_1$ buildup that they start making DNA immediately after removal of the blocker.[174] Exposing serum-activated T51B rat liver cells in confluent cultures to another naphthalene sulfonamide, W-13, at 10 h after serum addition, which is just before the expected onset of DNA replication, prevents initiation, but the cells will almost immediately start replicating their DNA if the inhibitor was to be removed 10 h later.[175] In other words, the liver cells can wait for 10 h with their huge replication factories ready to start production when cued by $Ca^{2+}$·CaM. What are the cells waiting for? Perhaps it

was for $Ca^{2+}$·CaM to unblock the transcription of the PCNA gene.[176,177] Whatever it is, it is not the same as the earlier external $Ca^{2+}$/CaR-dependent mechanism, which is too unstable to wait around for 10 h, which forces a buildup reset.

$Ca^{2+}$·CaM is also needed for DNA replication, and it has many targets in the nucleus that are involved in replication.[39,70] One of the targets is CaMKII, which would activate Cdk2·cyclin E and (or) Cdk2·cyclin A by phosphorylating and stimulating the Cdc25A CDK activator.[70,178] However, the CaMKII blocker, KN-93, prevents HeLa human cervical adenocarcinoma cells from initiating DNA replication seemingly *after* the surge of Cdk2·cyclin A activities has begun, which would indicate that $Ca^{2+}$·CaM-activated CaMKII stimulates something critical in or for the DNA factories after the CDKs' activation by Cdc25A (Fig. 1.2).[70,141] Another target is a 68-kDa $Ca^{2+}$·CaM-binding protein that surges along with CaM into the nucleus and binds to nuclear matrix-associated replication complexes.[179–182] The binding of $Ca^{2+}$·CaM·p68 to the replication factories on the matrix is associated with the stimulation of replication.[181,182] And CaM does seem to regulate both DNA polymerases α and δ.[183] Moreover, expressing a selectively CaM-binding inhibitor protein during the S-phase stops proliferation and reduces DNA replication, which indicates a continuing need for $Ca^{2+}$·CaM or CaM apoprotein during replication.[184] Yet, other $Ca^{2+}$·CaM targets might be the nuclear contractile machinery, consisting of actin, α-spectrin that binds actin to the inner leaflet of the nuclear envelope, caldesmon, α-fodrin, myosin light chain kinase, and myosin, which could be involved in orienting and configuring the replication foci and the $Ca^{2+}$·CaM-binding proteins that attach CaM to the nuclear matrix upon the onset of replication.[38,39,72] In particular, the surging $Ca^{2+}$·CaM would bind to caldesmon and release actin from its complex with caldesmon, which would allow actin to associate with myosin to form a contractile machine for nuclear and chromatin reconfiguring.

However, there is another $Ca^{2+}$-binding protein associated with the DNA-replication complexes that probably responds to the oscillating $Ca^{2+}$. This is the heavy chain of annexin II (also known as calpactin I or lipocortin II). The gene encoding annexin II is growth-regulated, and its product associates with four other components to make the primer-recognizing RF-C (replication factor-C) complex that, as we learned in the previous chapter, stimulates the assembly of the homotrimeric PCNA into a ring that clamps itself around the DNA strands and keeps DNA Pol δ progressively replicating the passing template strand.[36,185,186] Reducing the expression of annexin II in HeLa, 293, and 293T cells with antisense oligonucleotides reduces ongoing DNA replication, and immunodepleting annexin II in *Xenopus* egg extracts prevents DNA replication.[186,186a]

While we have been focusing on CaM flowing into the nucleus, we have missed something important happening on the cell surface. While CaM accumulates during the $G_1$ buildup in the cytoplasm of cells such as hepatocytes in the regenerating rat liver, K562 human leukemic lymphocytes, and T51B rat liver cells, its level *drops* (!) just before the onset of DNA replication.[187–189] Where does it go? Is it destroyed? Apparently not! The cell seems to dump it outside after the internal wave has crested despite the molecule not being addressed for secretion.[188] However, there are indications that it stimulates something on the cell's surface that is required to start replication. Thus, either adding $Ca^{2+}$ or CaM can stimulate $Ca^{2+}$-deprived T51B rat liver cells to start making DNA, and this DNA-synthetic response to $Ca^{2+}$ can be prevented by anti-calmodulin antibody, which is virtually certain not to get into the unpermeabilized cell let alone

reach the nucleus.[187,190,191] Proof of this for at least one type of cell is the ability of anti-calmodulin antibody or the CaM inhibitor W7 bound to agarose beads (to stop it from getting into the cell) to prevent the initiation of DNA replication and stop the proliferation of K562 leukemia cells.[188]

This external CaM action cannot be the same as the CaR-mediated action because the latter is blocked in the presence of reduced external $Ca^{2+}$ concentrations (e.g., 0.5 mM in the regenerating liver of the thyroparathyroidectomized rat) that would not be low enough to affect the formation of active $Ca^{2+}$·CaM complexes from any dumped CaM. Indeed, it appears that the progression to the CaM step depends on the external $Ca^{2+}$/CaR step. Thus, there is no wave of CaM or initiation of DNA replication when serum-deprived T51B rat liver cells are stimulated to start the $G_1$ buildup by fresh, low-$Ca^{2+}$ (0.01 mM) serum, but there is a CaM wave which crests and crashes between 12 and 16 h and the initiation of DNA replication between 14 and 16 h if $Ca^{2+}$ is added 4 h after serum.[187] Thus, it seems that the external $Ca^{2+}$/CaR-triggered step leads to the accumulation of CaM, some of which goes to the nucleus and the rest of which is dumped out of the cell and onto some surface receptor which drives an essential part of the buildup.

As a general rule, established largely by the results of experiments carried out at the National Research Council over the last 30 years, tumor cells greatly reduce or even lose their requirement for *external* $Ca^{2+}$ to build up to DNA replication.[36] However, since CaM and other $Ca^{2+}$-binding proteins are parts of the DNA-replicating factories, tumor cells simply could not be expected to be able to do without them. And, of course, they can't, as indicated by the malignant HeLa cell's need for CaMKII to start replicating DNA.[141] They are also needed by fibroblasts transformed by the constitutive hyperactivity of the avian sarcoma virus's 60-kDa v-Src protein tyrosine kinase. This wild hyperactive enzyme eliminates the need for adhesion-triggered integrin signals to restrain apoptogenesis and express CDKs such as Cdk2·cyclin A needed to trigger replication, and they can replicate DNA and proliferate normally and indefinitely in medium containing only 0.02 mM $Ca^{2+}$, which would silence a putative CaR and thus prevent normal cells from even starting replication.[36,188] However, they still need $Ca^{2+}$·CaM, because although they can proliferate rapidly and indefinitely with very little external $Ca^{2+}$ they become *extremely vulnerable* to the $Ca^{2+}$·CaM inhibitors, R24571and W7, just before (e.g., 2 h) DNA replication begins.[36,188,192]

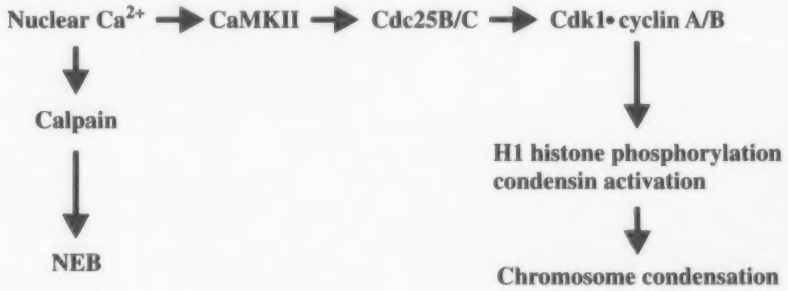
The $Ca^{2+}$ oscillations stop just after the onset of replication — the cell stops singing its "let's-replicate-DNA" song. When $Ca^{2+}$, $Ca^{2+}$·CaM, CaMKII, and the other $Ca^{2+}$-binding parts of the replication machines have done their jobs, phosphoCdk2·cyclin A accumulates in replication factories where it phosphorylates and activates both the initiator helicase that starts the firing of the first battery of replicons by preparing the DNA for being pulled along the assembly line by prying the DNA strands apart at the replication origins and the RP-A complex that then holds the single template strands apart for the polymerases. At the same time, the kinase has also de-licensed the new chromatids to prevent multiple rounds of replication by phosphorylating, and thus expelling the origin-bound, licensors, Cd6, and the MCM proteins. When the chromosomes are all replicated and there is no red light and wailing siren from an alerted $G_2$ checkpoint mechanism, the cell shreds the $G_1$/S-specific Cdk2 and starts expressing and using the mitotic Cdk1 which has been suppressed by Cdk2·cyclin A while the chromosomes were being replicated. It also starts expressing the last of the cyclins, the mitotic cyclin Bs.

## $Ca^{2+}$ in mitosis

The Cdk1·cyclin-driven breakdown of the nuclear membrane and all that goes with it are triggered by the strictly localized (punctate) release of $Ca^{2+}$ from thousands of endoplasmic reticular vesicles crowding around the nucleus (Fig. 2.3).[1,1a,15,193–197] In other words, the trigger is a shower of $Ca^{2+}$ "puffs" and "sparks," which would last for only a second at the most if they were away from the nucleus, but at this point they can last as long as 10 s because they are perinuclear and invade the nucleus.[197a] Preventing the $Ca^{2+}$ surges prevents prophase, while the $Ca^{2+}$-mobilizing caffeine or microinjected $Ins(1,4,5)P_3$ triggers premature prophase.[15,193–197] The phospholipase-C activity needed to generate $Ins(1,4,5)P_3$ does appear in the nucleus during the $G_2$ buildup.[197b] However, $Ins(1,4,5)P_3$ may not be the real physiological $Ca^{2+}$ liberator because it is freely diffusible, which the real liberator is not, and the PLC that would be needed to generate it is not in the membranes of the $Ca^{2+}$-storage/regulating vesicles in the prophase mitotic apparatus.[1] Instead, it could be leukotriene B4, which is confined to the vesicles along with the $PLA_2$ and the other enzymes needed to make it.[1]

The mediation through $Ca^{2+}$·CaM of the responses to the barrage of $Ca^{2+}$ from the thousands of perinuclear vesicles is suggested by the results of experiments with *ts*BN2 hamster kidney cells, which express a thermolabile mutant of the nuclear/chromosome-associated RCC1 protein,[197c] the disappearance of which at a non-permissive temperature such as 39.5°C triggers the premature initiation of prophase.[198] The disappearance of RCC1 is accompanied by a large CaM surge, the importance of which for the initiation of premature prophase is established by the ability of a $Ca^{2+}$·CaM blocker, W-7, to prevent this prophase response.[198] Exactly what $Ca^{2+}$·CaM ultimately does appears to be unknown. However, it does it by activating CaMKII.[193,194] When this remarkable multisubunit enzyme with its approximately 10 α- and β- or $\beta^1$-subunits is activated by $Ca^{2+}$·CaM, it autophosphorylates itself and becomes prolongedly active without further prompting by $Ca^{2+}$.[199] Thus, the shower of $Ca^{2+}$ transients triggers another, more

**Fig. 2.3.** How a surge of $Ca^{2+}$ into the nucleus from the nuclear envelope might trigger prophase. The surge produces $Ca^{2+}$·CaM complexes, which activate CaMKII, which in turn activates Cdc25BorC, the $G_2$-specific dual-function phosphatase that removes inhibitory phosphate from Cdk1 and thus activates the mitosis-specific CDKs. The CDKs phosphorylate the chromosomes' H1 histones, which causes the chromosomes to reconfigure themselves to admit the chromosome-condensing condensins, which have also been phosphorylated and "activated" by the CDKs. Meanwhile, the $Ca^{2+}$ spike has also stimulated the calpain protease, which starts breaking down the nuclear envelope (NEB).[1,39]



sustained, wave of CaMKII activity. But what does it do? It turns on the late-$G_1$-specific Cdc25C, a STY (serine/threonine/tyrosine) dual function phosphatase, which activates the Cdk1·cyclins A/B that in turn activate the chromosome-condensing condensins and phosphorylate H1 and H3 histones to reconfigure the chromosomes to receive the phospho-condensins that will condense them (Fig. 2.3).[90]

The $Ca^{2+}$ barrage may trigger nuclear envelope breakdown at least in part by directly stimulating the protease calpain (Fig. 2.3).[1a,39] In fact, inhibiting calpain blocks the $G_2 \rightarrow M$ transition at least in some cells.[200] This is supported by some evidence that, as mitosis approaches, calpain II relocates from the plasma membrane to the nucleus and its entourage of $Ca^{2+}$-loaded vesicles.[201] There, the calpain may participate in breaking down the nuclear envelope under the barrage of $Ca^{2+}$.

Another key contributor to the nuclear envelope breakdown is the $Ca^{2+}$-dependent PKC-βII, which is involved in the disassembly of the lamin network that lines the inner surface of the nucleus and provides "hitching posts" on the nuclear envelope for chromosomes. During the $G_2$ buildup, PKC-βII is translocated to the nucleus and plugged into the nuclear membrane alongside its target lamin B.[201a] Then, the membrane-tethered kinase is selectively activated when a product of nuclear membrane lipid breakdown, a specific type of nuclear phosphatidylglycerol, binds to its C-terminal region.[201a] The activated PKC-βII then triggers the disassembly of the lamin network and the release of chromosomes from the nuclear envelope by phosphorylating lamin B.[201b]

When the chromosomes have *all* assembled (congressed) at the mitotic spindle's mid-zone and the last of the cycle's checkpoint mechanisms (Fig. 2.1) that monitors and responds to signals from unattached kinetochores has been switched off, and the tension on the straining sister chromatids from the spindle fibers has reached a critical threshold, the Anaphase-GO! signal is given.[202] This is another $Ca^{2+}$ spike or spikes (Fig. 2.1).[39,195] It seems likely that Cdk1·cylin B induces the spike(s) and the $Ca^{2+}$ spills out of storage vesicles associated with the spindle.[1,1a,36] Once again, a $Ca^{2+}$ spike activates CaMKII, which, in turn, activates the spindle-restricted APC (anaphase-promoting complex) that contains a ubiquitin-conjugating E2 enzyme specific for mitotic cyclins and the securin that has been holding back the cohesin-cutting separin.[203–205] One result of this is the polyubiquitination of the mitotic cyclins which are marked for this and consequently the proteasome shredding by a conserved N-terminal motif known as the "destruction box."[36,205] The loss of the cyclin exposes the now naked Cdk1 catalytic subunit to further inactivation by having its $Thr^{161}$ residue dephosphorylated by type 2A protein phosphatase, which prevents the subunit from grabbing another cyclin. This cyclin destruction and the disabling of Cdk1 allow the chromosomes to decondense at telophase, but they are not needed for chromatid separation.[205] This requires the APC-driven destruction of the cohesins that have been tying the sister chromatids together[203,204], as well as the removal of residual DNA tangles by $Ca^{2+}$-stimulated, centromere-associated topoisomerase II.[206,207] The subsequent poleward ratcheting of the separated sister chromosomes along the spindle fibers by the "PacMan"-like action of their kinetochore engines is also driven at least in part by $Ca^{2+}$.[36]

Another important consequence of the CaMKII-triggered cyclin destruction and elimination of CDK activity is the enabling of the licensing of the new chromosomes for replication by the establishment of pre-replication complexes on the new replication origins by replacing the proteins that were expelled from the origin and eliminated as a

consequence of being phosphorylated by Cdk2·cyclin A during the passage of the DNA through the replication factory assembly line.[208] The new licensing proteins have accumulated in the cytoplasm, but they could not get at the chromosomes until the breakdown of the nuclear membrane, but even if they had somehow got into the nucleus before prophase they could not have attached themselves to the target origins because they would have been phosphorylated by the Cdk1·cyclins that took over from Cdk2·cyclin A.[157] However, as soon as a nuclear envelope is established in the daughter cells further access of cytoplasmic licensing factors is cut off.

$Ca^{2+}$ may also be involved in forming the new nuclear envelope during telophase. The fusion of pre-envelope vesicles (e.g., from the disassembled nuclear envelope in a mitosing *Xenopus* egg) into a new nuclear envelope requires the flux of $Ca^{2+}$ through the vesicles' Ins(1,4,5)$P_3$ receptor/channels.[39,209]

While all of this has been going on, the cell has been putting a belt of cortical actomyosin filaments around its middle. This belt has been kept from tightening prematurely by having its myosin motors turned off by the phosphorylation of certain residues in their light chains by Cdk1·cyclin B.[36] The motors are activated by the destruction of Cdk1·cyclin B and then started by the stimulation of their MLC (myosin light chain kinase) by the $Ca^{2+}$·CaM complexes generated by a final prolonged $Ca^{2+}$ surge.[36,195,205] The belt tightens and pinches the elongating cell into two daughters, each with licensed chromosomes awaiting instructions from their surroundings to cycle or differentiate, or from the spontaneous goading by inner voices to start cycling if they are malignant or are on their way to malignancy.

And now we must hurry on to skin keratinocytes and their $Ca^{2+}$ diffpoptosis.

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# 3
# Calcium and Keratinocyte Diffpoptosis

## Stem cells and cell stacks

The epidermis is maintained by the proliferation of basement membrane-attached cells in its basal layer. This cell production is balanced by *diffpoptosis*, a differentiation program of a tandemly changing transcriptome, the products of which are ultimately brought together by an apoptosis-like process only after the basal cells have detached from the basal lamina, stopped cycling, and stepwisedly accumulated the products of key $Ca^{2+}$-activated genes as well as $Ca^{2+}$-activated enzymes.[1] The keratinocytes are stacked layers of differentiating cells topped by horny-shelled corneocytes.[2–10] In the basal layer, there are clusters or patches of clonogenic stem cells lying on the basement membrane above the dermal papillae.[2–10] The clustered stem cells are tightly linked to each other by homodimers of the transmembrane Delta1 protein and the clusters are surrounded by a network of "transit amplifier" cells.[2–10]

The stem cell has a large nucleus and a small amount of cytoplasm. It is the only indefinitely self-renewable keratinocyte. Normally, it cycles only intermittently in its special niche (established by the combined efforts of many things among which are an adjacent epidermal Langerhans cell, the underlying dermal cells, and basal lamina components) to replenish the supply of transit amplifying cells while replacing itself.[3,9,10] Because of its small cytoplasm and initially limited load of the things needed for the $G_1$ buildup, it, like the similarly built small lymphocyte,[11,12] takes a long time (35-40 h in culture) to start the accumulation of the pocket protein-inactivating, cycle-starting Cdk4/6-cyclin Ds we met in Chap. 1, and to gear up for DNA replication and mitosis in the first cycle after receiving a "GO!" signal.[9] The stem cell has another peculiarity which is characteristic of tumor cells: it is stimulated to proliferate by the PKCs-stimulating, tumor-promoting TPA (12-*O*-tetradecanoyl phorbol-13 acetate) rather than differentiate as would its cycling progeny.[13,14] The stem cell makes types 5 and 14 keratins and has many proliferation-driving, $\alpha_2\beta_1$and $\alpha_3\beta_1$ integrins and strictly basal, keratin-linked hemidesmosomal $\alpha_6\beta_4$ integrins in focal adhesion complexes on its underside with which it binds itself tightly to the kalinin and laminin proteins in its basement membrane niche.[4–6,15–18] The larger, faster proliferating cells in the bottom layer of the cell stack are the morphologically serrated transit amplifying precursor cells, which express the ECaBP (Epidermal $Ca^{2+}$-Binding Protein), and they are attached less strongly to the basement membrane because they have many fewer $\beta_1$ integrins on their surfaces than the ancestral stem cell.[4,6,15–20] This difference between the surface densities of β1 integrins and adhesion strengths can be used to locate stem ("bright") cells and transit

amplifying ("dull") cells at the epidermal–dermal junction with fluorescently tagged anti-β1 subunit antibodies. The much stronger, integrin-mediated adhesion of stem cells to substrates such as collagen can be exploited to separate them from the transit amplifying cells in very small pieces of residual normal skin and use them to repair burns and other injuries.[5,6,10,11]

The stem cell has a considerable, if not unlimited, proliferative potential that depends at least in part on its expression of telomerase (a reverse RNA transcriptase that protects the chromosomes' ends from progressive shortening at each round of replication by adding to their G-rich, 3′-ends (Fig. 3.1).[21–23] Therefore, it can produce large colonies in culture.[5] An unlimited proliferative potential and the many rounds of its replication that occur over many years demands that the stem cell genome also be protected by the vigilance of the products of the mismatch repair genes that Kinzler and Vogelstein have called the genome's "caretakers."[24] However, the more loosely attached, although bigger and faster cycling, transit amplifying and differentiation-committed cells have only a limited proliferative potential and consequently can form only much smaller colonies in culture.[5] They seem to have some kind of cycle counter which starts out with five on it. After five cycles, the number drops to 0 in the counters of the 32 progeny (even in those of an isolated *cultured* transit amplifier cell), which are programmed to switch off their cell cycle genes, dismantle their cell cycle engines, stop cycling, start stratifying, and set out on the diffpoptotic road leading to the maintenance of the permeability barrier and dead, keratin-packed corneocytes. But these "terminal" cells are not irreversibly "terminal" because they can be "immortalized" (i.e., reacquire a stem cell's proliferative potential) by expressing the adenoviral E1 protein, which can switch off or reset the cycle counter.[25]

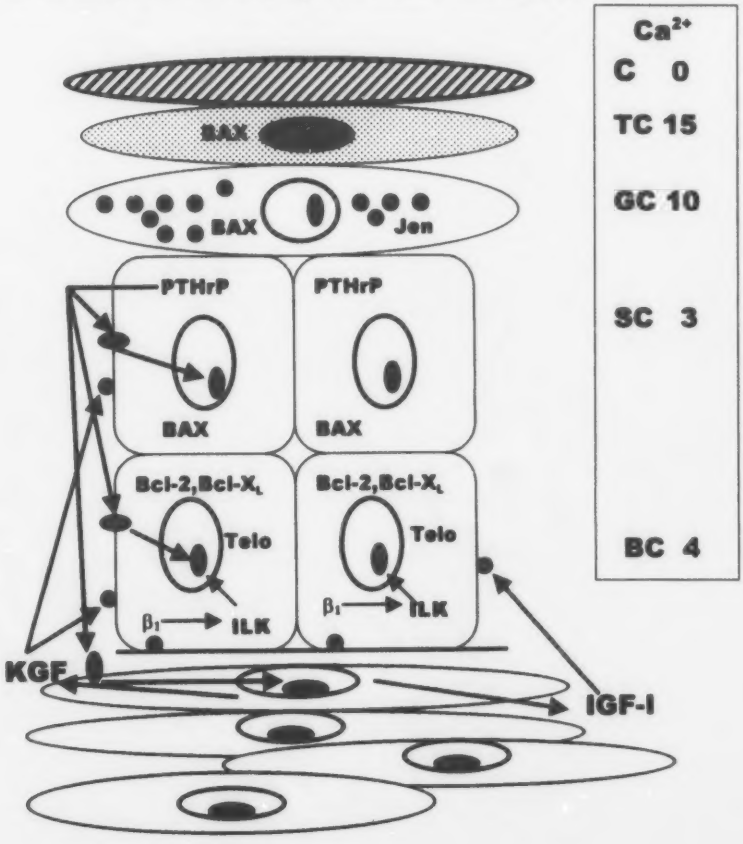
As we learned in Chap. 1, normal cells such as the basal cells need the signals from $\beta_1$ integrin receptors clustered in focal adhesion complexes of several signaling enzymes and the tyrosine kinase receptors for soluble growth factors such as TGF-α to express c-Myc, make Cdk4/6·cyclin Ds to inactivate pocket proteins and thus activate E2F target genes, which results in surges of cycle-driving Cdk2·cyclin E and Cdk2·cyclin A.[5,11,25a] Also as we learned in Chap. 1, the cytoplasmic domains of the β1 subunits of these adhesion-activated integrins are attached to the 59-kDa serine/threonine protein kinase, ILK (Integrin-Linked Kinase) (Fig. 3.1).[26–28] ILK is activated when it contacts PtdIns(3,4,5)$P_3$ made in the cell membrane from PtdIns(4,5)$P_2$ by PtdIns-3Ks (type I phosphoinositide-D3 kinase). PtdIns-3K is stimulated when GTP·Ha-Ras binds to its 110-kDa catalytic component or when the phosphotyrosines of integrin-clustered/activated FAKs (Focal Adhesion complex protein tyrosine Kinases) are inserted into the NSH2 and CSH2 pockets of the enzyme's 85-kDa regulatory component.[25a,26,28–31]

Signals from the membrane-associated ILKs stimulate the translocation of β-catenin into the nucleus to suppress the expression of the gene for the β-catenin-binding E-cadherin and to stimulate the expression of the genes for cell cycle drivers such as cyclin D1 and c-Myc and, through c-Myc, the cyclin D2 gene and the gene for the cyclin Ds' partner, Cdk4, in the cycle-starting CDKs, Cdk4·cyclin D1 or D2.[31a–36] The PtdIns(3,4,5)$P_3$-activated ILK and PtdIns(3,4,5)$P_3$-activated PDK(phosphoinositide-dependent protein kinase)-1 kinase give a "double whammy" to the cell by phosphorylating and activating PKBs (protein kinases B) attached to membrane PtdIns(3,4,5)$P_3$ by their PH (pleckstrin homology) domains, which also stimulate the

**Fig. 3.1.** The skin and its $Ca^{2+}$ gradient (the values are from human skin and expressed as mmol/kg dry weight). Basal keratinocytes (BC) need a low external $Ca^{2+}$ concentration and signals from $\beta_1$ integrins attached to the basal lamina to proliferate. These signals stimulate ILK kinase and prevent the destruction of β-catenin, which stimulates cell cycle genes such as c-*myc* and suppresses E-cadherin expression. The stem cells and their basal transit amplifying progeny avoid apoptosis by making Bcl-2 or Bcl-$X_L$ proteins and suppressing the Bax killer protein. They also express telomerase (Telo), which prevents their chromosomes' telomeres from shortening with each round of replication.

Reducing β1 integrin expression causes the cell to lift off the basal lamina and generate a $Ca^{2+}$ surge that stops proliferation and starts the cell making the things needed to become a corneocyte. The emerging spinous cell (SC) stops expressing Bcl-2, Bcl-$X_L$, and Telo and starts expressing Bax. It also starts making full-length PTHrP and chops it into fragments. The PTHrPs stimulate the dermal fibroblasts to express and secrete IGF-I and KGF. IGF-I promotes the basal cell survival and proliferation. KGF also promotes basal cell proliferation by inducing the cells to express TGF-α and prevents the spinous cells with their high density of KGF receptors from prematurely initiating apoptosis.

The final stages of differentiation in the granular cells (GC) are driven by signals from the CaRs ($Ca^{2+}$ receptors) activated by a sudden rise in the external $Ca^{2+}$ concentration when the cell nears the head of the transepidermal $Ca^{2+}$ gradient. At the top of the gradient, the CaR signaling in the transitional cells (TC) triggers the apoptosis-like mechanism that converts the cells into the corneocytes of the horny layer (or stratum corneum [C]).



translocation of β-catenin into the nucleus as well as fend off apoptosis (by stimulating Bcl-2 production and inactivating the pro-apoptosis BAD protein) and increase the activity of the E2F transcription factor, which, as we learned in the previous chapter, targets and switches on the genes for key DNA-replicating enzymes.[26,28,37,38]

β-Catenin does two very important, yet very different, things, depending on where the cell is in the epidermal stack. First, it couples the cytoplasmic C-terminal tails of the $Ca^{2+}$-binding/activated E-cadherin with α-catenin, vinculin, α-actinin, and F-actin to make intracellular cables that tie the suprabasal spinous cells together.[39–41] However, this locking of β-catenin into cables must be stopped by ILK and PDK-1 if basal cells are to proliferate. What is most important for a *cycling* basal cell is β-catenin's role as a *transcription factor* for cycle driver genes. Suppressing E-cadherin expression and cable-making with ILK increases the amount of free β-catenin that can be shipped into the basal cell's nucleus (in a Ran-independent manner)[42] to join an "architectural" transcription factor such as one of the LEFs (lymphocyte enhancer-binding factors) / TCFs, which is bound to specific DNA motifs and has sharply bent the promoters/enhancers of the target genes to bring together widely separated DNA motifs to make a platform for assembling the components of transcription-initiating or inhibiting complexes.[34,39,43] Without β-catenin, LEF/TCF associates with CBP (CREB-binding protein) and the Groucho and CtBP (C-terminal TATA box-binding) co-repressor proteins in a complex that, for example, shuts off the c-*myc* gene.[44,44a] However, when β-catenin surges into the nucleus it replaces Groucho to make a β-catenin·LEF/TCF complex on the promoter DNA, which then assembles an active transcription–initiation complex with RNA polymerase II.[40,43–44a] In anticipation of things to come in the next chapter, colon cells climbing up the crypt wall have been found to make another protein besides E-cadherin that arranges for destruction of free β-catenin rather than just locking it up. This protein is the tumor-suppressing APC (adenomatous polyposis coli).[32,39,40,44–46] It binds to and reconfigures a protein known as *axin*.[44a,46,47] The re-configured axin acts as a "marriage broker" which brings β-catenin and GSK(glycogen synthase kinase)-3β to the same "table."[44a,46,47] GSK-3β then phosphorylates β-catenin, a 'kiss of death' which marks it for ubiquitination and dumping into the proteasome shredder we met in the previous chapter.[44,44a,48] But both ILK itself and the PKB it activates phosphorylate and thus inhibit GSK-3β.[26,38] This saves β-catenin from the shredder and enables it to get into the nucleus to make cycle-driving β-catenin·LEF-1 complexes.[27,44,44a,49]

The $\beta_1$ integrins also link to the Shc adaptor protein via caveolin in the caveolar cell membrane pouches to provide a docking site which has been velcroized with phosphorylated tyrosine residues to collect a cluster of SH2/SH3-containing components, such as Grb·mSos complexes, that contribute to the sustained adhesion-dependent signaling that keeps the cell cycling and prevents it from killing itself prematurely with the apoptogenic cascade of caspase proteases discussed in the previous chapter.[25a,49a,50] These signals stimulate the cells: to load their membranes with the key phospholipase-Cγ1 signaling substrate PtdIns(4,5)$P_2$ needed for signal generation by the growth factor velcroceptors; to make critical stage-specific CDKs; to stimulate the proliferogenic, gene-activating, Ras/Raf-1-triggered, MAP kinase cascade alongside growth factors; to reconfigure the cytoskeleton to enable the cell to respond to growth factors.[51–55] Perhaps surprisingly, the integrins also bind and activate the receptor protein tyrosine kinases for soluble growth factors such as EGF/TGF-α and PDGF.[56] In fact, integrin-induced clustering can activate

PDGF receptors even without their ligand, PDGF, and integrin-induced co-clustering can also activate EGF/TGF-α receptors and enhance the EGF-dependent activation of the MAPK cascade.[56] Of course, these β1 integrin-induced clusterings of the soluble growth factor kinases amplify PtdIns-3K activity and the activity of the all-important β-catenin-sparing, PtdIns(3,4,5)$P_3$-activated ILK and PKB.

However, the proliferating basal cells must protect themselves from a premature activation of the apoptogenic mechanism by the proliferation-driving c-Myc·Max complexes and signal byproducts, such as reactive oxygen intermediates if there should be any unschedeuled interruption of the stream of survival signals from the integrins and their clustermates. They do this by making anti-apoptosis protector proteins in response to signals from their $\beta_1$ integrins. The stem cells make the anti-apoptosis Bcl-2 protector that we met in the two previous chapters, while their progeny may supplement it or replace it with the related Bcl-$X_L$ protector protein.[57,58] They are also smart enough not to express the dangerous, pro-apoptosis Bax protein.[57,58] Indeed, Bcl-2 may be a stem cell marker.[59]

Adhesion-induced clustering of the cytoplasmic domains of integrin $\beta_1$ subunits in focal adhesion complexes produces integrin-talin complexes, which in turn form complexes with the pivotal 125-kDa FAK protein tyrosine kinase.[51–55,60] FAK components of the integrin:talin-induced oligomers then velcroize each other by transphosphorylating their $Tyr^{397}$ residues located in their [-Tyr-Ala-Glu-Ileu-] motifs, which are specific docking sites for c-Src or other members of the Src family such as c-Fyn.[52] An approaching c-Src protein tyrosine kinase then shoves a FAK phospho-$Tyr^{397}$ into its SH2 pocket. The now docked and locked Src then phosphorylates, and fully activates, the FAK by phosphorylating Tyr residues in other motifs, such as the [-Tyr-Met-X-Met-] motif that specifically binds the NSH2 and CSH2 pockets of the 85-kDa regulatory subunit of PtdIns-3K, the resulting activity of which is needed to prevent apoptosis and promote proliferation by producing PtdIns(3,4,5)$P_3$ to stimulate ILK, PDK-1, PKB, PKCs δ, ϵ, η, and ς or to mediate the stimulation of the MAP protein kinase cascade by growth factors and the C-terminal [-$Tyr^{925}$-Glu-Asn-Val-] motif that specifically binds the SH2 pocket of the Grb-2 adaptor.[25a,26,52,61] Grb2 in turn binds with its SH3 group to the mSos Ras GDP/GTP exchanger protein in the cell membrane. This triggers the replacement of GDP with GTP to produce active membrane-bound GTP·Ras complexes. These Ras complexes can then directly stimulate the neighboring PtdIns-3Ks and trigger the $G_1$ buildup-driving Raf-1/MEK/MAP kinase cascade. Other signaling enzymes that are directly or indirectly turned on include the following: $PLA_2$, with a resulting arachidonate surge and production of eicosanoids; PLC-γ1, which breaks PtdIns(4,5)$P_2$ down into $Ca^{2+}$-mobilizing Ins(1,4,5)$P_3$ and PKCs-stimulating diacylglycerols; and the Rho-mediated stimulation of PtdIns(4)P-5 kinase that loads the membrane with PtdIns(4,5)$P_2$ with which the activated PI-3K makes the ILK and PKB-stimulating PtdIns(3,4,5)$P_3$.[28,37,51–54,56] A very important upshot of this adhesion-driven cascade is the expression of several genes, one of which is the gene for cyclin A (but not the genes for cyclin D, cyclin E, Cdk1, or Cdk2), which, as we have seen in the previous chapters, is a component of the Cdk2·cyclin A protein kinase that is generated specifically at the end of the $G_1$ buildup and surges into the nucleus to work throughout the S phase in the replication factories activating replication origins.[55,56,60,62–64] The adhesion signals may also suppress the expression of the growth-arrest-specific gene, *gas*1, and its membrane-associated

cycle-suppressing protein product.[65,66] As we shall soon see, switching off the β1 integrin signals streaming into the basal cell triggers the diffpoptosis program.

It must be assumed that the basal keratinocyte's β1-integrin-primed cycle is driven in the same way by the same machinery, cycle clocks, waves of CDKs and $Ca^{2+}$ transients that drive the cycles of other cells we met in the previous chapters. It also includes the continuous expression and actions of the Id(inhibitor of differentiation)-1, -2, -3 proteins that suppress the activation of the diffpoptosis program by blocking the action of bHLH (basic Helix–Loop–Helix) transcription factors.[66a] Signals from the 7-TMD receptors for β-adrenergic agonists and the EGF/TGF-α velcroceptors, a pack of paracrine factors (extracellular matrix-bound bFGF, hepatocyte growth factor/scatter factor, IGF-I, IGF-II, KGF [keratinocyte growth factor, a.k.a. FGF-7]) secreted by dermal fibroblasts, and probably the most important of all, the keratinocytes' autocrine/paracrine TGF-α, drive keratinocyte proliferation by stimulating the parade of stage-specific CDKs and suppressing the expression of the diffpoptosis drivers. However, the effectiveness of these agents depends on the membrane's stores of substrates for receptor protein kinases such as PtdIns(4,5)$P_2$ and the expression of, among many things, the cyclin A gene by adhesion-activated β1 integrins.[1,51,54,63,64,67,68] The very important TGF-α is active both as the tip of a large transmembrane precursor sticking out of the cell surface, which stimulates proliferation by binding to EGF/TGF-α velcroceptors on adjacent cells, a "*juxtacrine*" activation mechanism, and as the soluble small "paracrine" TGF-α peptide, which when snipped off the precursor diffuses toward the velcroceptors on neighboring cells.[69,70] Keratinocytes also need a small amount of external $Ca^{2+}$, such as 0.05 mM,[1] to cycle, but as we shall soon see, cycling stops and different parts of the diffpoptosis program start when the external $Ca^{2+}$ concentration rises above a certain level.

## The $Ca^{2+}$ gradient

A single, major, if not *the* major, diffpoptosis driver is the striking, steep epidermal $Ca^{2+}$ gradient where the $Ca^{2+}$ level at the granular/horny layer interface is at least 4–5 times higher than in the basal and spinous layers (Fig. 3.1).[71–73] In the human epidermis, the total calcium contents of the basal and spinous zones are about 3–4 mmol/kg dry weight, but the content jumps to about 10 mmol/kg dry weight in the granular layer and finally to 15 mmol/kg dry weight in the lower horny layer (Fig. 3.1).[71] This gradient is an ancient (probably established around $350 \times 10^6$ years ago along with the parathyroid glands as two of the sine qua nons for the invasion of dry land) and elegant way of ensuring that the parts of the diffpoptosis program are triggered at the right times and places in the epidermal stack and that the vital permeability barrier is set up and maintained. But how is the gradient loaded at the top when the only source of $Ca^{2+}$ in a dry land-dwelling animal, the blood, is at the *foot* of the gradient?

The gradient develops relatively late in mouse (day 19) and rat (day 20) fetal development as the fetus nears the time when it will need the double protection of the permeability barrier and the horny layer to face the air and insults.[65] It is established along with the permeability barrier in a stratified horny layer.[72] A clue to how it might be done lies in the observations of Menon et al.[74,75] who used an oxalate-pyroantimonate probe to precipitate and localize $Ca^{2+}$. $Ca^{2+}$ was found in the cytosol, mitochondria, and the nuclei of some basal and spinous cells but *not* in the intercellular spaces in either layer. The

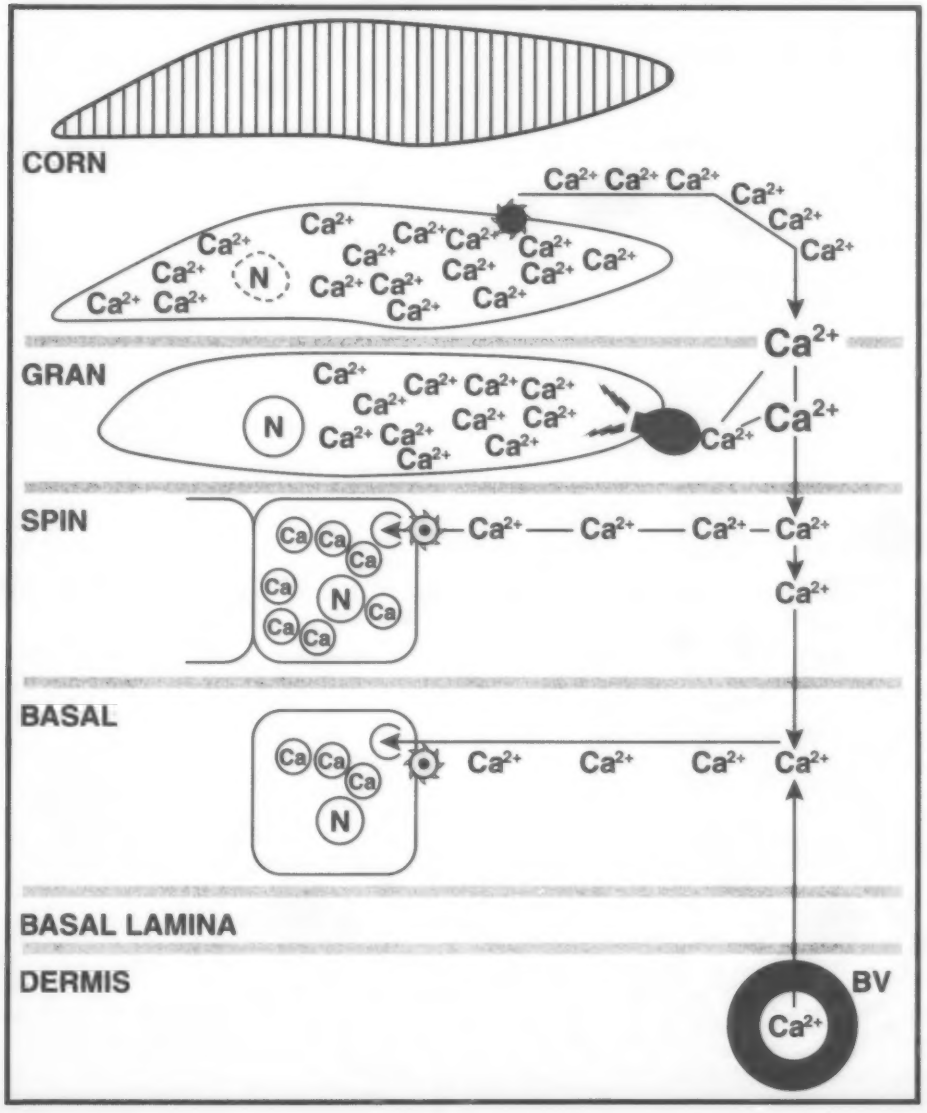
heightened total $Ca^{2+}$ level in the granular layer observed by Grundin et al.[71] points to this being the place where *extracellular* $Ca^{2+}$ starts driving diffpoptosis. Indeed, in the experiments of Menon et al.[74,75] *inter*cellular $Ca^{2+}$ first became detectable in the lower granular layer ($Ca^{2+}$ had disappeared from the nuclei and was restricted to the mitochondria [maybe an indication of the start of the apoptogenic megachannel formation in the mitochondrial inner membranes] and to the limiting membranes and discs of the lipid-rich lamellar bodies[76]) and then rose dramatically in the mid-granular layer. This was followed by a surge of intracellular $Ca^{2+}$ in upper granular cells. By contrast, most horny-coated corneocytes had no demonstrable $Ca^{2+}$ — they had pumped out the $Ca^{2+}$ they had accumulated in the upper granular layer before they destroyed their plasma membranes and their $Ca^{2+}$ pumps in the final apoptotic frenzy. This distribution with the highest $Ca^{2+}$ levels in the granular and lower horny layers and low levels in the upper horny layer has also been seen by Vicanova et al.[77] in human epidermis cultured in a serum-free medium with vitamins and lipids, which was optimal for rebuilding the permeability barrier.

Although the rapidly cycling basal keratinocytes are exposed to a very low *external* $Ca^{2+}$ concentration, they have substantial amounts of cytosolic and even nuclear $Ca^{2+}$ (which probably reflects the transport of $Ca^{2+}$ into the nucleus, which we learned in the previous chapter occurs at the end of the $G_1$ buildup) with which to drive their cycling. They may use their ECaBP to get this $Ca^{2+}$ by sucking up and sequestering $Ca^{2+}$ coming up from the blood and seeping down from the granular and lower horny layers and thus produce the low *external* $Ca^{2+}$ concentration in the basal layer needed for optimal integrin activity (integrin receptors do need a small amount of $Ca^{2+}$ to assume their preactivation configurations, but it is the high-$Ca^{2+}$-inhibitable action of $Mg^{2+}$ and (or) $Mn^{2+}$ that is then needed for the receptors' active configurations[78]). At the same time, there is a large enough internal $Ca^{2+}$ store to produce the $Ca^{2+}$ spikes and oscillations that trigger the major events of the cell cycle as well as enough external $Ca^{2+}$ to enable the cell to progress through the brief, possibly $Ca^{2+}$·CaR-dependent, critical later stage of the $G_1$ buildup.[1,79] The accumulated $Ca^{2+}$ is ultimately expelled from upper granular cells' internal stores by signals from the CaRs ($Ca^{2+}$-responsive polyvalent cation receptors)[80–84] to trigger the cornifying apoptosis, and all of it is then pumped out of the developing corneocytes by $Ca^{2+}$·CaM-activated membrane pumps before the cell membrane is destroyed and replaced by the horny envelope. This prevents a potentially steady and massive leakage of $Ca^{2+}$ from the body in shedding corneocytes. Thus, all of the $Ca^{2+}$ collected by the cells during their journey from the bottom to the top of the stack is dumped out to trickle down into the intercellular spaces of the granular layer and activate the $Ca^{2+}$-receptors on the upcoming granular cells as it goes (Fig. 3.2). This recycling $Ca^{2+}$ is, therefore, a *downwardly diffusing diffpoptosis driver* (Fig. 3.2).

## What triggers diffpoptosis?

It appears that the process starts with the progressive loss of surface β1 integrins, the reduction of ILK and PKB activities that keep the transit amplifiers cycling as they migrate or are pushed along the basement membrane away from the stem cell niche.[6,15,16] Indeed, when the volume of integrin/ILK and PKB signaling drops below a critical level, $Ca^{2+}$ spills out of internal stores, which probably triggers the influx of external $Ca^{2+}$ through SOCs (Stores-Operated non-specific $Na^+/Ca^{2+}$ Channels[83]). The resulting

**Fig. 3.2.** How could the epidermis make the top-down $Ca^{2+}$ gradient when the $Ca^{2+}$ must come from dermal blood vessels (BV) at the bottom of the gradient? One possibility is this "*load-up-then-dump-out*" mechanism in which the cell accumulates and locks up $Ca^{2+}$ to maintain a low intercellular concentration while moving from the basal layer through the spinous (SPIN) layer into the lower granular (GRAN) layer. Midway into the granular layer, the cell's CaRs are activated (as indicated by the sparks from a representative CaR) by a dramatically increased intercellular $Ca^{2+}$ concentration. This signaling produces intracellular $Ca^{2+}$ surges that drive the conversion of the cell into a keratin-packed corpse. But before it dies, the cell uses its $Ca^{2+}$-activated pumps to dump the accumulated $Ca^{2+}$ into the intercellular space to maintain the head of the gradient.



internal $Ca^{2+}$ surge (it must be understood that the external $Ca^{2+}$ concentration even in the basal layer is much higher than the nanomolar free $Ca^{2+}$ level inside the cell) shuts down the production of the Id-1, -2, and –3 diffpoptosis suppressors and stimulates the expression of the gene coding for the spinous type 1 keratin, which, in turn, starts the production of the type 10 keratin when it reaches a critical level. This scenario is suggested by the observation of Li et al.[66a,84] that suspending primary BALB/c mouse keratinocytes *in a low-$Ca^{2+}$ (0.5 mM) medium* triggered internal $Ca^{2+}$ surges resulting from the emptying of $Ca^{2+}$ stores plus a flow of $Ca^{2+}$ into the cell. These suspension-triggered $Ca^{2+}$ surges stopped proliferation and triggered the expression of the keratin 1 gene (which has a $Ca^{2+}$-responsive enhancer), the keratin 10 gene, the loricrin gene, the filaggrin gene, and the accumulation of their products despite an overall *reduction* of protein synthesis.[85] $Ca^{2+}$ shock (from raising the external $Ca^{2+}$ concentration from 0.05 to 1.4 mM) in mouse keratinocytes suppresses TGF-β1 expression and stimulates TGF-β2 expression and secretion,[86] but detachment stimulates TGF-β1 expression and secretion by human keratinocytes.[87] Thus, a detachment-triggered $Ca^{2+}$ surge stimulates the production and secretion of autocrine/paracrine cycle-stopping TGF-β1 or TGF-β2 and other diffpoptosis-related proteins. The detachment-triggered signal might also activate the *gas*1 gene and the accumulation at the cell membrane of its cycle-suppressing protein product as occurs in colon cells.[66] However, the downregulation of ILK expression and the silencing of the proliferogenic, apoptosis-preventing FAK, ILK, and PKB kinase signaling should be enough, and is indeed enough, to stop cycling by preventing the appearance of cyclin A and the DNA replication-triggering Cdk-2·cyclin A protein kinase complexes at the end of the $G_1$ buildup.[88] Thus, for example, detaching murine keratinocytes from their substrate drops the cyclin A level, which as would be expected from the role of Cdk2·cyclin A in driving DNA replication stops the cells in S-phase.[88] The resulting detachment-triggered $Ca^{2+}$ surge might also reduce the anti-apoptotic Ras activity induced by the proliferogenic signals from the integrin-receptor clusters.[89]

## $Ca^{2+}$ and diffpoptosis in the unreal world of the culture dish

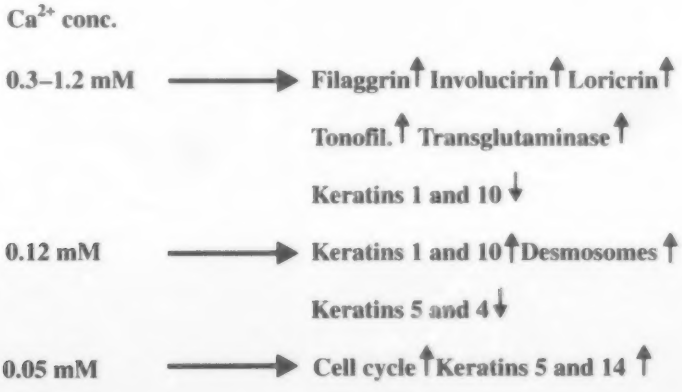
Where and how $Ca^{2+}$ drives diffpoptosis in the real world of the skin are hard to see in the large body of information from experiments on cultured primary keratinocytes or keratinocytes of established lines in the unreal world of the culture dish. The differentiation program in cultured primary human foreskin keratinocytes can be triggered either by shocking the cells with a large increase in the external $Ca^{2+}$ concentration or by inducing $Ca^{2+}$ signals by cell–cell contact or detachment from the culture dish. Thus, raising the $Ca^{2+}$ concentration in the medium removes a major barrier to diffpoptosis by suppressing the expression of the anti-apoptosis, Bcl-$X_L$ protein, and stimulating the production of the pro-apoptosis Bax protein.[58] Cells grown in a low-$Ca^{2+}$ (0.07 mM) medium start making the horny envelope constituent involucrin (from the Latin involucrum, envelope) and envelope-making transglutaminases to get ready for making a cornified envelope only one week *after* they start touching each other and stop proliferating when their culture becomes confluent.[90] By contrast, cells cultured in a shock medium containing 1.2 or 2.4 mM $Ca^{2+}$ make involucrin and transglutaminases *before* confluence. This enables them to start making, or trying to make, horny envelopes without prior contact. In other

words, the $Ca^{2+}$ shock signals were enough to switch on the appropriate genes to start and then drive differentiation.[90]

In most experiments on cultured cells, the $Ca^{2+}$ concentration in the medium is simply raised from a proliferation-supporting 0.05 to 1.0 or 1.2 mM or higher. This $Ca^{2+}$ shock triggers an internal $Ca^{2+}$ surge and a highly compressed (or "zipped" to use a computer term) set of events, ranging from proliferative shutdown to cornified envelope formation.[1,84,85,91,92] While a $Ca^{2+}$ shock can trigger diffpoptosis in cultured cells, the preparations for cornification and barrier maintenance in the real world of the skin would have to be started at or near the basal→spinous transition by internal $Ca^{2+}$ surges triggered by a drop in adhesion-induced integrin-orchestrated signaling in the presence of a low external $Ca^{2+}$ concentration simply because the $Ca^{2+}$ concentration in the gradient does not start rising until the cell is halfway through the granular layer (Figs. 3.1 and 3.2).[71,74,75,77]

There is evidence that the different stages of diffpoptosis can be selectively triggered or suppressed by different levels of $Ca^{2+}$ shock (Fig. 3.3). Thus, the shock given by raising the $Ca^{2+}$ concentration in the medium from 0.05 to 0.12 mM causes mouse keratinocytes to switch off their cell cycle genes and the genes coding for the small, cycling-compatible keratins 5 and 14 and to switch on the genes for the larger keratins 1 and 10 and other events such as surface display of the $Ca^{2+}$-configured/activated E-cadherins (at the expense of the β1- integrins) and associated desmosome assembly responsible for the cell–cell-adhesion and desmosome-attached heterodimeric keratin 1/10 tonofibrillar bundles that are the morphological hallmarks of spinous cells (Fig. 3.3).[1,15,92,93] The stronger shock from raising the $Ca^{2+}$ concentration in the medium to 0.3 or 1.2 mM, or triggering an internal $Ca^{2+}$ surge by emptying internal $Ca^{2+}$ storage vesicles by exposing

**Fig. 3.3.** Parts of the diffpoptosis program in cultured keratinoctyes can be selectively stimulated or stopped by raising the $Ca^{2+}$ concentration in the culture medium by certain amounts. While these in vitro findings cannot be directly extended to the skin, they may be models for, and thus mimic, the responses to a rising intensity of $Ca^{2+}$ signaling in vivo, first by detachment from the basal lamina, then by autocrine and paracrine factors produced in the spinous layer, and finally by $Ca^{2+}$ receptors (CaRs) in the granular and lower corneum layers, which trigger the stepwise initiation of events and inhibition of preceding events leading from basal cell to corneocyte.



cells to an inhibitor of the storage vesicles' inwardly directed $Ca^{2+}$ pump, thapsigargin, shuts down the keratins 1 and 10 genes and stimulates the expression of the genes of the granular cell/corneocyte parts of the diffpoptosis program that code for filaggrin, involucrin, loricrin, and the keratinocyte-specific $TG_K$ (the keratinocyte-specific transglutaminase) (Fig. 3.3).[85] Raising the $Ca^{2+}$ concentration from 0.05 to 1.0 mM causes the cells to bypass earlier stages and directly become partially cornified corpses. The need for a $Ca^{2+}$ gradient explains why keratinocytes stratify and differentiate better in floating, air-exposed cultures (liquid medium/air interface similar to skin) than in submerged cultures.[1,10,90] The floating cultures can establish the physiological, or near-physiological, $Ca^{2+}$ gradient and permeability barrier. However, in submerged cultures the $Ca^{2+}$ concentration would equalize with the concentration in the medium. From these observations on isolated cells, it seems that epidermal diffpoptosis can be driven stepwise by superficially similar (although undoubtedly both temporally and spatially very different) $Ca^{2+}$ signals from different receptors to different targets appearing at different stages of the program.

## $Ca^{2+}$ and diffpoptosis in the real world of the skin

Are these observations on cultured keratinocytes relevant to keratinocyte differentiation in the real world of the skin? All they tell us is that a $Ca^{2+}$ shock can stop keratinocytes from proliferating and can differentiate to a varying extent, depending on the strength of the shock. However, there are some striking differences between the ultimate products of differentiation in cultures and skin, one of which, for example, is an inability of $Ca^{2+}$-shocked cultured keratinocytes to make the rigid envelopes of the mature horny cells in the skin.[94] So let us try to decompress or "unzip" the responses of the shocked cells and put them together with observations on keratinocytes in the skin to try to see how a newborn transit amplifying keratinocyte can produce a pile of water-repellant, lipid-coated horny cadavers. From the outset, we must keep in mind that during this diffpoptotic journey specific mRNA transcripts appear when the cell enters a layer only to vanish when the cell enters the next layer/stage, but their many protein products stay on and accumulate to collaborate with the next batch of proteins to ultimately produce corneocytes and maintain an effective permeability barrier. In other words, pulses of stage-specific mRNAs drive the buildup of the many things needed to make a corneocyte.

The diffpoptosis program starts on the border of a stem cell cluster where cells have lowered their expression of the transmembrane Delta1 protein that binds the stem cells together in the body of the cluster.[10,94a] The signal to become a "transit amplifying" cell is given to the border cell when its transmembrane Notch receptors bind to Delta1 proteins on neighboring cells of the "transit amplifying" network.[10,94a] As a result of the Delta1–Notch signal, the cells loses its "immortality": its cycle counter (registering 5) has been switched on. Its cycling accelerates, but it has lost the ability to produce large clones, and its descendants will stop cycling when their cycle counters register 0. Armed against apoptosis by the PKB-promoted expression of the anti-apoptosis Bcl-2 and Bcl-$X_L$ proteins and the inhibition by PKB of the pro-apoptotic ICE-like proteases[37,58], it is still tightly bound to the basal lamina by its β1 integrins which densely decorate its surface.[6] The decision to start a new cycle is governed by the number on its cycle counter, the

volume of flow of agents through gap-junctions (intercellular channels made of hexamers of connexin-43) connecting its interior to those of overlying spinous and adjacent basal cells[95–102] and by various growth factors such as IGF-I and KGF secreted by underlying PTHrP-stimulated dermal fibroblasts. KGF also causes the cell to make the autocrine proliferogen TGF-α.[103] (However, we shall learn further on that it is the spinous cell and *not* the cycling basal cell that has the greatest density of KGF receptors on its surface.) Transit amplifying cells pick up other factors from the underlying dermal cells in micropinocytic vesicles formed between the hemidesmosomes of their serrated basal surfaces.[104]

If the decision is "GO!," the cell will continue the cycle countdown by starting the $G_1$ buildup to DNA replication and mitosis. It will of necessity express the Id-1, -2, and -3 diffpoptosis-suppressors and the master replication-related transcription factors such as c-Myc with its vast array (as many as 198) of target genes such as those for CAK, cyclin D1, and E2F1, which results in the production and activation of Cdk4·cyclin D1 that starts the $G_1$ buildup by inactivating the Rb-family "pocket proteins," which releases E2F1 from E2F·pocket protein complexes to start the cresting and crashing waves of CDKs that drive the expression of replication-related genes.[35,66a,104a–106] Of course, it will generate the $Ca^{2+}$ spikes that start the $G_1$ buildup and trigger DNA replication, mitotic prophase, and finally the metaphase·anaphase transition. Besides their ILK-, PKB-, and β-catenin-mediated upregulation of c-*myc* and other cycle-driving genes, the signals from the β1 integrins stimulate the cell to express the Bcl-2 and Bcl-$X_L$ proteins and suppress the expression of pro-apoptosis Bax protein to prevent it from prematurely killing itself through the actions of reactive oxygen intermediates and mitochondrial $Ca^{2+}$ cycling generated by growth factor receptor activities and the apoptogenic arm of c-Myc action.[1,35,37,49,55,107–111] Near the end of the $G_1$ buildup, the cell starts its replication-initiating $Ca^{2+}$ oscillations.[1,79] The integrin and IGF-I signals stimulate the expression of the cyclin A gene.[55,62–64] Cyclin A now downregulates and replaces cyclin E to form the Cdk2·cyclin A protein kinases that will terminate the Cdk2·cyclin E/E2F·DP-1-driven expression of the genes for the DNA-replicating enzymes and at the same time activate DNA-replication origins and start and then maintain the sequential firing of the replicon batteries during the DNA-synthetic S phase.[112,113] After replicating its chromosomes, the cell will get rid of its Cdk2·cyclin A protein kinases and make the Cdk1·cyclin A and Cdk1·cyclin B protein kinases that, together with $Ca^{2+}$ transients, bursts of phosphatase activity, and help from the proteasome shredder, will drive the cells into and through mitosis.

## *The first step: from basal cell to spinous cell*

Depending on the balance of the "GO!" and "NO GO!" signals, the new cells will start new cycles and move, or be pushed, further away from the ancestral cluster on the basal lamina. As they do so, they express the ECaBP and start to reduce their complement of β1 integrins, which progressively weakens their attachment to the basal lamina and reduces the activities of PtdIns-3K, ILK, and PKB.[4–6,20,21,26,37] Along with this, the cells stop making the transmembrane Delta1 protein and thus stop the signaling from the Delta1·Notch complexes that triggered the transition from stem cells to transient amplifying cells.[94a] This fading of signaling from the adhesion complexes and Delta1·Notch

creates a subpopulation of basal cells that have begun changing their transcriptome for next stage of the program as indicated by the appearance of keratins K1 and K10.[4] Important factors in the maintenance of their proliferative activity may be their expression of TGF-β1, which specifically stimulates the expression of the basal cell-specific keratins 5 and 14,[114] a low-level production of the inhibitor, TGF-β2, and its retention in the cytoplasm.[115,116]

When the signaling from β1-integrins drops below a critical level, probably before the cell has actually lifted from the basement membrane, the tyrosine kinase receptors / FAK / Ras-driven PtdIns-3K activity drops, PtdIns(3,4,5)$P_3$-dependent ILK and PKB signaling stops, and there is an internal $Ca^{2+}$ surge resulting from the discharge of internal stores and a coupled influx of $Ca^{2+}$ through SOC/CRAC cation channels in the cell membrane.[30,31,84] *This is likely what silences the Id-1, -2, -3 protein diffpoptosis suppressors, starts the diffpoptosis program, and sets the cell on the road to the permeability barrier, the head of the $Ca^{2+}$ gradient, and ultimately a horny death.*

Before the transcriptome can shift to start the diffpoptosis program, the cell cycle-driving Cdk-cyclins are silenced by members of the Cip/Kip family of CKIs (cyclin-dependent kinase inhibitors). As we learned in the previous chapters, these CDK inhibitors stop the cell cycling in several different ways: by inhibiting the Cdk2-cylin E protein kinase that otherwise would stimulate the expression of the replication enzymes; by inhibiting the Cdk2-cyclin A protein kinase that would activate batteries of origin-attached DNA replication complexes throughout the S-phase; and by inhibiting PCNA, the accessory DNA strand-clamp for the replication factories' DNA polymerase-δ. The first of these CKIs to go into action is the multifunctional $p21^{Cip1/WAF1}$. Things start with the p53(TP53)-*independent* (as in intestine[117] p53(TP53) which normally stimulates p21 expression actually *crashes*), p300 protein-mediated, activation of the $p21^{Cip1/WAF1}$ gene's $Ca^{2+}$-responsive promoter.[118–121] p21's job is to knock the Cdk-cyclins out of action, which prevents the inactivation (by hyperphosphorylation) of the pRb cycle suppressor, and stops proliferation. As we shall also see in the intestine,[117] p21 is not involved in starting diffpoptosis, but it must be removed of as soon as it has done its job, otherwise it would block the expression of at least one of the major diffpoptosis-related genes, involucrin, possibly through the interaction of p21-CDK complexes with the gene's promoter.[119] Therefore, after the CDKs have been silenced, and cycling has stopped, p21 is dropped into the ubiquitin-proteasome shredder to allow diffpoptosis to start.[119] Then, p57 and another multifunctional member of the Cip/Kip family of CKIs, $p27^{Kip1}$, surge upwards.[120–122] But $p27^{Kip1}$'s job is very different from that of p21. Despite being a CKI, it is not involved in cycle-stopping (p21 has already done this), but instead it is needed to start the diffpoptosis program.[122]

Detachment-triggered $Ca^{2+}$ signals also stimulate the expression and secretion of TGF-β1 in human keratinocytes,[87] but probably suppress TGF-β1 expression and secretion and stimulate the synthesis and secretion of TGF-β2 in mouse skin.[86,114] A surge of TGF-β1 or β2 could contribute further to the proliferative shutdown by stimulating the later expression of the Cdk 4/6-specific inhibitor, $p15^{INK\ 4b}$, which displaces another inhibitor, $p27^{Kip1}$ from Cdk4 and Cdk6 that can then bind to Cdk2 and block the $G_1$ buildup.[118,123,124] We might expect that the detachment signal would stop c-*myc* gene expression, but surprisingly it seems that it does not.[125–127] Indeed, a continued expression of c-*myc* in a cell expressing its death machinery without other $G_1$ drivers to provide

survival signals could start prematurely the apoptotic arm of diffpoptosis.[109] Moreover, detachment alone is dangerous and would trigger apoptosis in any normal keratinocyte unprotected by Bcl-2, Bcl-$X_L$, or an activated mutant Ha-Ras protein.[30]

Daughters of the penultimate descendants of a transit amplifying cell will probably not load their DNA replication origins with ORC·Cdc45·MCMs pre-initiation complexes that have been licensed for replication by RLF-B: they have lost their license for replication. Also, as we learned in Chap. 1, the rapidly cycling transit amplifying cells would have DNA-synthetic complexes known as synthesomes (the operational parts of the huge nuclear matrix-bound chromosome replication factories), which they retain even if they should temporarily stop cycling.[128,129] This complex contains CaM, DNA polymerases α and δ, primase, replication factor C (with its $Ca^{2+}$-binding 36-kDa annexin II subunit), replication protein A, helicases, poly(ADPribose) polymerase, PCNA (proliferating cell nuclear antigen), DNA ligase, and topoisomerases I and II.[128,129] However, if a keratinocyte is like other terminally differentiating cells, one of the first things it does when it starts diffpoptosis is to stop making parts for the replication factories, such as DNA polymerase-α, replication factor C, and PCNA, and get rid of existing factory parts.[129] Along with this is a shutdown of the expression of telomerase that protected the chromosomal ends from end-erosion by replication over the generations from the ancestral stem cell (Fig. 3.1).[21–23]

Although basal keratinocytes have functional death machinery, their high levels of Bcl-2 and Bcl-$X_L$ (induced by ILK-activated PKBs and the PKB's inhibition of pro-apoptotic ICE-like proteases) keep it switched off (Figs. 3.1 and 3.2).[37,56] Progressive loosening of attachment, the fading of adhesion-induced signals, the loss of the Ha-Ras/PtdIns-3K suppression of apoptosis, and the resulting $Ca^{2+}$ surge could probably lift the Bcl-2/Bcl-$X_L$-imposed barrier to the mobilization of the protease components of the death machinery, such as the mitochondrial-derived caspase (ICE [pro-interleukin 1β converting enzyme] cysteine protease[s]), as does the detachment of mammary epithelial cells.[56,130] Detachment also downregulates Bcl-2 expression and upregulates Bax protein expression so that the death machinery can eventually be switched on by the loudly signaling CaRs ($Ca^{2+}$-activated divalent cation receptors) when the cell reaches the head of the $Ca^{2+}$ gradient in the upper granular layer.[58,131–135] Another possible consequence of the loss of β1-adhesion complex signaling and the lack of CDK activity is the accumulation of the hypophosphorylated Rb protein, which has been found to trigger apoptosis in some cells.[56] This suicidal detachment-triggered apoptosis is known as *anoikis* (from the Greek word for homelessness, hence *homelessness-induced programmed cell death*), which we first met in Chap. 1.[136]

Another reason for anoikis might be the detachment of DCCs, receptors belonging to the immunoglobulin superfamily that were first found to be deleted in the later stages of colon carcinogenesis and subsequently found to be expressed in navigating neurons and the basal layers of many epithelia including skin.[137] Neurons use their DCC receptors to bind to the netrin landmarks that guide their growth cones to their targets.[137] Disconnecting DCC receptors from their binding sites in the extracellular matrix triggers aniokis in normal, but not in neoplastic, cells.[138]

Clearly, detachment from the basal lamina and a changing transcriptome usher in a perilous period for the seemingly defenseless new spinous cell. However, the cell defends itself from anoikis by greatly increasing its complement of KGF receptors and

making PTHrP (see below) to stimulate the underlying dermal fibroblasts to make KGF.[139] It is this KGF from the fibroblasts that takes over from the departing Bcl-2, Bcl-$X_L$, and the Ha-Ras/PtdIns(3,4,5)$P_3$/PDK-1-stimulated PKB to suppress the premature onset of such terminal diffpoptotic events as the activation of membrane-associated transglutaminase, horny envelope construction, and chromosome chopping during the spinous stage.[32,121]

The detachment-triggered $Ca^{2+}$ signal stimulates the expression and shipment into the nucleus of transcription factors for the first set of differentiation-driving genes. For example, the signal stimulates the expression of the gene for the membrane-anchored, 22 5-kDa, S100-like, $Ca^{2+}$-binding, CLED (Calcium-Linked Epithelial Differentiation) protein.[139a] In another example, a cycling basal cell has a lot of C/EBPβ (a bZIP-family CCAAT enhancer-binding protein) and some CHOP9C/EBP-homologous protein factors in its cytoplasm but little in its nucleus.[140] However, the detachment-triggered signal causes C/EBPβ and CHOP to move into the nucleus.[140] This signal stimulates through the gene's $Ca^{2+}$-responsive promoter, the first of the differentiation events, the expression of the spinous keratin-1 gene (located in the cluster of type II keratin genes in human chromosome 12q13[141]).[142,143] When keratin-1 reaches a critical level, it triggers the expression of keratin 10, the gene for which has been primed by the incoming C/EBPβ binding to a specific site in the gene's 5′-regulatory region.[1,140,143a] The $Ca^{2+}$ signal may also enhance C/EBPβ's transcriptional activity by phosphorylating the protein with a $Ca^{2+}$-CaM-activated protein kinase such as CaMKIV.[144,145] The cell stops making the $Ca^{2+}$-transporting ECaBP protein, which will not be needed when the cell reaches the upper levels of the $Ca^{2+}$gradient.[19] At this point, the cell is still expressing β1 integrins but the intracellular transport of newly synthesized β1 integrins to the cell surface for installation in the membrane stops as the new proteins are held at the endoplasmic reticulum by the reticulum's $Ca^{2+}$-binding protein, calnexin; and β1 receptors already on the surface are endocytosed and delivered to the lysosomes for destruction.[146,147]

At the same time, detachment and the internal $Ca^{2+}$ surge do the key things for starting diffpoptosis. When the cell was attached to the basal lamina, the β-catenin·LEF-1 transcription complexes generated by its β1-integrin-activated ILK suppressed the expression of the E-cadherin gene, which has an inhibitory LEF-1-binding motif in its regulatory region.[40,148] The detachment of the cell and the consequent silencing of ILK and the PKBs results in the dephosphorylation and activation of GSK-3β. GSK-3β then phosphorylates free β-catenin, which sends it to the proteasome shredder via ubiquitination. This destruction of β-catenin shuts down key cycle-driving genes and turns on the E-cadherin gene.[7,26–29,32,36,37,39,43–49] The emerging cadherins now suppress the expression of the β1 integrins and ILK.

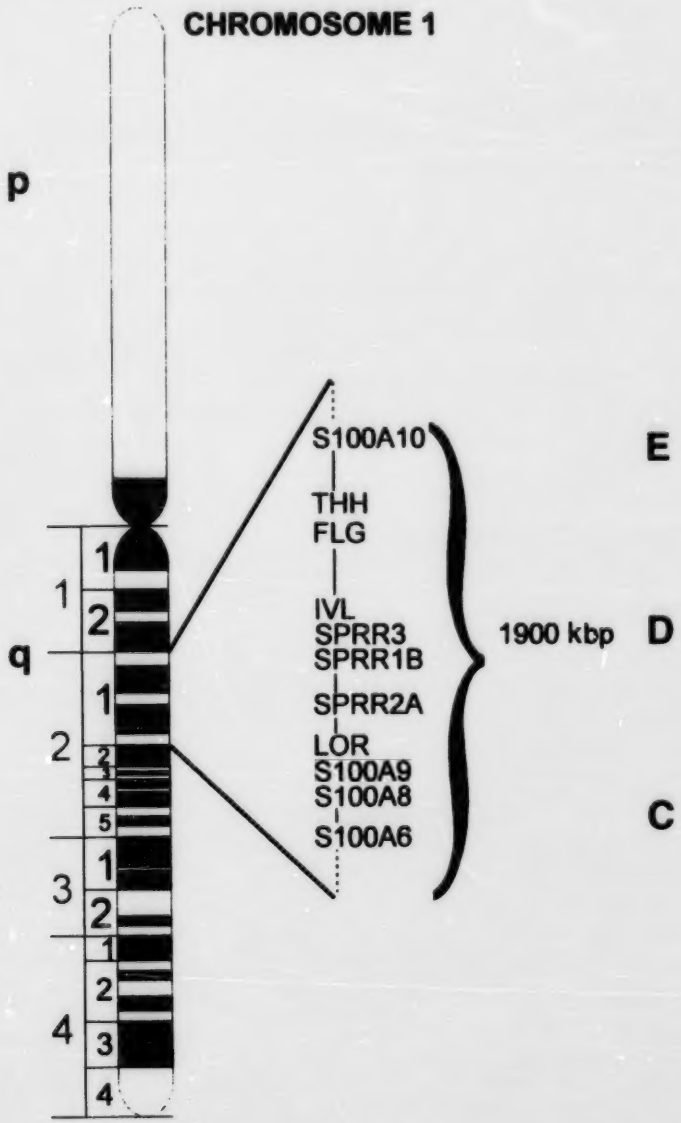
The $Ca^{2+}$ signal causes the protein tyrosine kinase Fyn (or in its absence Src) to move to the cell membrane where it promotes the formation of E-cadherin–F-actin cables by phosphorylating the E-cadherins and the β-catenin and γ-catenin (plakoglobin) linkers.[149] Outside the cell, the emerging cadherins, shifting into their optimal adhesive configuration when their five $Ca^{2+}$-binding motifs (the "cadherin repeats") meet the relatively high (with respect to internal) external $Ca^{2+}$ concentration, cause the lifting cell to bind to the E-cadherins protruding from its neighbors' surfaces.[146,150–152] If keratinocyte E-cadherins behave like those of EMT/6 mammary carcinoma cells, they may add yet another cycle-suppressor by increasing the activity of the protein kinase inhibitor, $p27^{Kip}$,

which reduces cyclin D1 production and thus "activates" Rb by preventing its hyperphosphorylation.[153] However, Alani et al.[118] have found that Cdk4 complexes do not change during the differentiation of human keratinocytes in the culture dish. But most important for our $Ca^{2+}$ story is the fact that it is at this point in the diffpoptosis program that *external* $Ca^{2+}$ becomes a major player in the shutting down and dismantling of the cell cycle engine because the $Ca^{2+}$-driven interlinking of the emerging E-cadherins to those on adjacent cells triggers the locking of β-catenin into intracelluar E-cadherin·α-catenin·β-catenin·vinculin·α-actinin·F-actin cables, which, of course, keeps the β-catenin from getting into the nucleus and driving cell-cycle gene expression.

The cell now becomes a keratin-making machine that produces larger differentiation-compatible keratins such as the spinous-specific types 1 and 10 that will make up more than 85% of the total protein of the ultimate horny cadaver.[93,154] The keratins 1 and 10 combine into 1·10 heterodimers, which form a network by building onto the preexisting network of keratin 5·14 heterodimers left over from the proliferating basal stage.[154–156] The $Ca^{2+}$-dependent interlinking of cells by the emerging cadherins sends a signal to stimulate the expression of new proteins such as desmoglein, a cadherin that is specifically associated with desmosomes, which are the cellular "pop-rivets" that attach the fledgling spinous cell to its neighbors.[1,40,150,152,157] Each desmosome consists of cell–cell-interlinking extracellular domains of the desmosome-specific cadherins (the 100–120-kDa desmocollins and 130–150-kDa desmoglein) whose cytoplasmic tails in turn bind to a dense cytoplasmic plaque (consisting of a core of 240-kDa desmoplakin I, 215-kDa desmoplakin II, and 82-kDa plakoglobin bordered by the huge 680-kDa desmoyokin) into which the keratin 1·10 heterodimers loop and bind to specific plaque proteins to form what is effectively a resilient supracellular structure that interlinks the cells of the epithelial sheet to provide great flexibility combined with a strong resistance to rupture.[40,157] The cell starts making the full-length, 106-kDa, keratinocyte-specific transglutaminase ($TG_K$) and the 68-kDa rod-shaped involucrin, both of which it sends to the plasma membrane for use much later to make the horny envelope.[94,158–162]

The promoter of the involucrin gene on chromosome 1q21-22 in humans, like the $Ca^{2+}$-responsive promoter of the gene for the cycle-stopping $p21^{Cip/WAF1}$, is co-activated by p300.[120] The human involucrin gene has a $Ca^{2+}$-responsive, 5′-regulatory element between nucleotides –2456 and –1272 and a PKCs-responsive, AP-1 transcription factor-stimulated regulatory element between nucleotides –159 and –1.[163] Between these two promoter regions, there is a –651 to –160 transcription silencing region that interacts with a spinous-specific Fra-1(or -2)·Jun D AP-1 heterodimer as well as the YY1 transcription silencer.[163,163a] It would be reasonable to guess that YY1 attached to the silencer sequence represses the expression of involucrin in proliferating keratinocytes, but the silencer is inactivated when cell cycling stops and the detachment-triggered $Ca^{2+}$/PKCs/AP-1(Fra-1(or -2)·Jun D) signal stimulates the gene's $Ca^{2+}$-responsive regulatory region. The cell also starts making the small proline-rich SPRR1 protein (the human homolog of cornifin in rabbits), one of a family of 12 small proteins which, when the time comes, will cross-link the various larger proteins of the cornified envelope.[160,161] The SPRR1 (cornifin) gene, like the involucrin gene, is another member of the EDC (epidermal differentiation complex), a remarkable cluster of genes on the q21-22 region of human chromosome 1 (Fig. 3.4).[164–166]

**Fig. 3.4.** The epidermal differentiation gene complex (EDC) on human chromosome 1q21-22. The members of this gene cluster can be divided into three groups: genes coding for cornified envelope constituents (IVL, involucrin; LOR, loricrin; SPRR, small proline-rich proteins); genes coding for macrofilament-producing keratin intermediate filament cross-linkers (FLG, profilaggrin; THH, trichohyalin); and genes coding for the $Ca^{2+}$-binding S100 proteins. Their tight clustering has enabled the profilaggrin and trichohyalin genes to fuse with the neighboring S100 genes and to each acquire two S100-like $Ca^{2+}$-binding motifs in their regulatory elements and with them a responsiveness to $Ca^{2+}$ signals from the granular cell's divalent cation receptors. This figure from Whitfield.[1]



The members of this gene cluster encode involucrin, loricrin, profilaggrin, SPRR1B, SPRR2A, SPRR3, S100A6, S100A8, S100A9, S100A10, and trichohyalin (Fig. 3.4).[164–166] Their products can be divided into three groups: the cornified envelope components involucrin, loricrin, and the SPRR proteins; the macrofilament-making keratin intermediate filament cross-linkers, profilaggrin and trichohylalin; and the S100 $Ca^{2+}$- binding regulators. This intimate mingling of cornified envelope-making genes with the S100 genes in human and other animals (Fig. 3.4) has enabled two of the key players in keratinocyte diffpoptosis, profilaggrin and trichohyalin (which are next to S100A10 and S100A11), to borrow S100 coding sequences and thus become responsive to $Ca^{2+}$ signals. Both of these proteins have acquired two N-terminal S100 $Ca^{2+}$-binding motifs, which are followed by tandem repeats characteristic of the cornified envelope proteins.[165–170] Although tightly clustered on chromosome 1, the EDC genes are not coordinately regulated: they are turned on at different points in the diffpoptotic program, and some, such as the SPRR genes, are controlled by a different set of transcription factors and are expressed in different regions of the skin.[164]

The human spinous cell also starts making the protease-inhibitor keratolinin (Gk: *keratos*, horny; *lininos*, inner surface cover) or cystatin A, which will play an important supporting role in the run-up to cornified envelope formation.[160] As the cell nears the top of the spinous layer, it starts accumulating the 300–500-nm ellipsoidal lamellar bodies, each containing a battery of still inactive hydrolases (e.g., acid phosphatase, proteases, lipases, and glycosidases) and packed with a much-folded ("accordioned") lipid membrane the components of which (glucosylceramides, free sterols, phospholipids) will later be released from the cell, processed, and made into the lipid permeability barrier.[72,76] The cell also starts making cholesterol sulfatase separately in order to produce the cholesterol sulfate required to "glue" the cell to its neighbors until desquamation.[76,104,171] At this point, the expression of gap junction proteins in mouse spinous cells switches from connexins 26 and 43 to connexins 31 and 31·1.[95] This restricts junctional permeability and the transjunctional chattering and exchanges between themselves and underlying basal cells by replacing the basal cell-type gap junctions with junctions that still transfer small metabolites such as nucleotides and amino acids as effectively as the basal cell gap junctions, but transfer larger molecules such as neurobiotin, carboxyfluorescein, and Lucifer yellow less effectively than the basal cells' junctions.[95]

At this point, we must address the roles of TGF-β2 and the IGF-II-selective IGFBP-6 (IGF-binding, hence inhibiting, protein-6) in starting the differentiation program. Basal cells must not make or secrete these potent autocrine/paracrine cell cycle stoppers. However, suprabasal cells in the skin are known to start secreting TGF-β2 into intercellular spaces and continue into the upper layers.[116] Experiments on human HaCat keratinocytes indicate that these cells constitutively secrete *latent* TGF-β2 in a low-$Ca^{2+}$ (0.03 mM) medium, but secrete *active* TGF-β2 when the medium contains 1 mM $Ca^{2+}$.[116] A high external $Ca^{2+}$ concentration plus a high cell density also stimulates the secretion of IGFBP-6.[116] While these factors could account for the inhibition of the proliferation of cultured keratinocytes by high-$Ca^{2+}$, they probably are not key proliferative inactivators of suprabasal cells in the real world of the skin because detachment from the basement membrane and the cessation of β1-integrin signaling should be more than enough to stop cycling. Moreover, neither the total nor the intercellular $Ca^{2+}$ concentration rises until the cell reaches the granular layer (Figs. 3.1 and 3.2).[71–75]

Besides loading up with heavy keratins, welding itself to its neighbors with desmosomes and E-cadherins, and starting to make the lipids of the permeability barrier, micellar granules, and future cornified envelope components (e.g., SPRR1 and involucrin), the neophyte spinous cell now upregulates the expression, and production, of the potent signaler, PTHrP (Fig. 3.1).[172,173] PTHrP appears to have been involved in making skins from the beginning of vertebrate evolution because it is expressed in the skins of the jawless lamprey, lungfish, and sharks,[174] which suggests that it or its progenitor was already working in the epidermis of the amphioxus-like, proto-vertebrate, *Pikaia*, and the more recently discovered ancient chordates *Haikouella* and *Yunnanozoan* that swam in the warm equatorial Cambrian seas more than 0.5 Ga ago.[174a,175]

Unlike the blood-borne endocrine PTH, with which it shares 8 of its first 13 N-terminal amino acids and a functionally identical receptor-binding/activating configuration in its first 34 amino acids, PTHrP does not circulate in the blood of *normal* adult animals and humans but is locally produced in many tissues where it operates as an autocrine/paracrine factor.[176,177] Depending on the type of producer cell, the PTHrP gene can be transcribed into one of three mRNAs encoding a mature 139-, 141-, or 173-amino acid protein with 5–7 endoprotease target motifs that enable it to be processed, like pre-pro-vasopressin or proopiomelanocortin, into smaller bioactive products with different receptors and different functions.[176,177] Some of these fragments with the intact PTHrP N-terminus will bind in order to then activate the type I PTH/PTHrP receptors on the producer cell's surface, the surfaces of its neighbors, as well as on the surfaces of the more distant, although still local, dermal fibroblasts (Fig. 3.1).[178]

PTHrP is a polyprotein, other fragments of which may activate different receptors on the surfaces of both the producers and other local cells. Stem cells do not express their PTH/PTHrP receptor genes.[179] Expression starts in the early, slowly cycling transit amplifying basal cells.[179] The functioning, differentiation-modulating product of this gene is not the usual type I receptor found on other cells such as dermal fibroblasts, osteoblasts, and proximal kidney tubule cells.[178] In other cells, the conventional type I receptor is coupled to both the $G_s$ protein that activates cyclic AMP synthesis by adenylyl cyclase and to one of the $G_{q/11}$ proteins that activates membrane-associated phospholipase-Cβ, which breaks PtdIns(4,5)$P_2$ down into diacylglycerols that stimulate membrane-associated PKCs and Ins(1,4,5)$P_3$, which in its turn triggers the release of $Ca^{2+}$ from the internal stores bearing the Ins(1,4,5)$P_3$ receptors we met in the previous chapter. But the normal keratinocyte's PTH/PTHrP receptor is the product of a different gene. Keratinocytes do not express their gene for the conventional type 1 receptor (PTHR1), and the receptor they do express activates a phospholipase-Cβ but not adenylyl cyclase despite an ample supply of functional $G_s$ proteins as indicated by the ability of the β-adrenergic agonist isoproterenol to induce the cells to strongly stimulate adenylyl cyclase.[180–184] However, they have not irretrievably lost the ability to make the conventional receptor because during the course of their "immortalization" human RHEK-1 keratinocytes began expressing conventional PTH/PTHrP, adenylyl cyclase-stimulating PTH/PTHrP receptors.[185]

There could be another reason for this unconventional responsiveness of keratinocytes to PTH. HEK (human embryonic kidney)/W cells seem to respond to hPTH-(1-34) like keratinocytes.[186] Their adenylyl cyclase is not inactivated, although as in keratinocytes, it can be strongly stimulated by forskolin or prostaglandin $E_2$. However, they

do respond with a biphasic release of $Ca^{2+}$ from internal stores, which is not triggered by phospholipase-C-generated Ins(1,4,5)$P_3$. The $Ca^{2+}$ release is triggered by a different, ryanodine-sensitive mechanism. This "peculiar" response is somehow due to the HEK/W cells having a very low (in fact not measurable) PTHR1 density. Raising the receptor density to 350 000/cell to produce HEK/T cells can replace or override this response with the conventional one consisting of a strong stimulation of adenylyl cyclase and a biphasic, Ins(1,4,5)$P_3$-triggered release of $Ca^{2+}$ from internal stores. Does this mean that the keratinocyte response to PTH might be due, or partly due, to a low conventional receptor density rather than a differently coupling receptor?

Other fragments containing the PTHrP molecule's C-terminal T(Thr)$^{107}$-R(Arg)$^{108}$-S(Ser)$^{109}$-A(Ala)$^{110}$-W(Tryp)$^{111}$ motif or even just the small TRSAW fragment itself stimulate membrane-associated PKCs activity without affecting adenylyl cyclase.[187,188] Do keratinocytes express different receptors for N-terminal PTH/PTHrP fragments and fragments bearing the TRSAW motif?[188,188a] Or do they express a receptor which is activated by both kinds of fragment? We eagerly await the answer to these questions.

We don't know whether keratinocytes in the skin make C-terminal PTHrP fragments with the (*107–111*) TRSAW motif. They might do so because the spinous cells in a reconstructed organotypic culture of human foreskin appear to chew up the holoprotein and specifically make a fragment that binds an antibody to PTHrP's *34–53* mid-region.[189] The signals from the receptors stimulated by TRSAW-bearing PTHrP fragments are extremely potent *two-way modulators* of keratinocyte proliferation.[188] Thus, when BALB/MK mouse keratinocytes have stopped proliferating because of EGF withdrawal from their medium, one 30-min incubation each day in a medium containing 10 nM TRSAW (and the brief burst of membrane-associated PKCs activity it causes) followed by return to the orginal EGF-free medium will at least *partially replace* EGF and stimulate them to start, and then continue, proliferating.[188] On the other hand, if the cells are already cycling in an EGF-containing medium, *only one* 30-min exposure to 10 nM TRSAW is enough to stop them proliferating for at least 5 days, perhaps by starting the cycle-stopping part of the differentiation program.[188] Primary adult human keratinocytes are even more sensitive to TRSAW. As little as $10^{-14}$ M TRSAW stops EGF-driven proliferation, and exposure to $10^{-10}$ M maximally stimulates the EGF-deprived human cells to start proliferating.[188]

PTHrP is a rather special signaler like bFGF and PDGF.[190,191] Besides being just a conventional surface receptor-activating autocrine/paracrine factor operating through a surface receptor, it is an "intracrine" factor that is selectively transported into the nucleus and particularly to the dense fibrillar core of the nucleolus where it affects various nuclear functions such as ribosomal RNA production, which would increase the cell's protein-producing capacity.[165–167] The mature, unprocessed protein has a bipartite nuclear localization (NLS) motif in its *87–107* region (i.e., just upstream from the TRSAW region).[192–194] The NLS of PTHrP binds to the 90-kDa importin-β, and the resulting complex docks on nucleoporins in the nuclear pore complex, which starts the GTP-fueled transport of the PTHrP through the pore and into the nucleoplasm.[195–197] But how does nascent PTHrP get to the nuclear border to be ferried into the nucleus? Surely this must be the wrong way because PTHrP has a signal sequence which should, and in fact does, direct it into the endoplasmic reticulum and from there through the Golgi apparatus where it is addressed, packaged for secretion, and eventually dumped into the

extracellular space. When the secreted PTHrP attaches to a receptor, the PTHrP·receptor complex can be endocytosed and then delivered rapidly to the nucleus.[196] However, the protein can also be delivered directly to the nucleus without leaving the cell.[196] This direct delivery might be due to the signal sequence being altered during translation in which case the protein would bypass the endoplasmic reticulum and be shipped into the nucleus.[194,194a]

What triggers PTHrP expression? The short answer at the beginning of 2001 is — *We don't know*. It seems to require the low-$Ca^{2+}$ concentration characteristic of the basal and spinous layers, and its production could be stimulated by one of the factors secreted by the dermal fibroblasts. It could be induced by the detachment-triggered $Ca^{2+}$ signal. This possibility is suggested by the $Ca^{2+}$·CaM-mediated stimulation of PTHrP gene expression in the $K^+$-depolarized cerebellar granule cell by a surge of $Ca^{2+}$ through voltage-opened L-type $Ca^{2+}$ channels in the cell membrane beside or directly on top of the nucleus.[198] It could be induced by autocrine TGF-β2, which, as we have seen, is specifically expressed (at least in mouse skin) and secreted suprabasally, because it has been shown that TGF-βs strongly stimulate PTHrP production by human foreskin keratinocytes and cells of a squamous carcinoma-derived cell line.[199,200]

It follows from these observations that the autocrine/paracrine 139-173-amino acid PTHrP polyprotein holomolecule and (or) one or more of its several differently bioactive fragments, which are induced in the emerging spinous cell possibly by the detachment-triggered $Ca^{2+}$and $Ca^{2+}$·CaM surges, activate the keratinocyte's unconventional PTH/PTHrP receptor(s). The PLC-β-activating signals from the surface receptors would trigger more $Ca^{2+}$ and $Ca^{2+}$·calmodulin surges and a surge of membrane-associated PKCs' activity, which together could stop proliferation and stimulate the production of involucrin, keratins 1 and 10, and transglutaminase as has been observed in the cells of $Ca^{2+}$-shocked primary cultures of human keratinocytes and cultures of BALB/MK mouse keratinocytes.[180,181,188,201] The mediation of these responses to $Ca^{2+}$ shock by PTHrP is indicated by the ability of the suppression of PTHrP expression in human HPK1A keratinocytes by antisense RNA to accelerate proliferation and prevent the shock from inducing the production of keratins 1 and 10, involucrin, and transglutaminase and from stimulating the expression of the involucrin gene.[201] At the same time, those endogenous PTHrP molecules or PTHrP molecules from neighboring cells with the (*87–107*) NLS motif would be shipped to the nucleolus on endocytosed receptors to increase ribosomal RNA production to enhance the translation of the transcripts of the genes for differentiation-related proteins. Of course, PTHrP might also be a transcription factor or cofactor.

An important clue to the function of PTHrP is the ability of the phosphorylation of $Thr^{85}$ near the NLS to prevent the protein's import into the nucleus.[201a] $Thr^{85}$ is targeted by CDKs containing Cdks 1 and 2, which results in PTHrP being *excluded* from the nucleus, starting from the Cdk2·cyclin E-driven stage of the $G_1$ buildup to the Cdk1·cyclins A/B-driven $G_2$/M.[201a] Thus, PTHrP would not be expected to affect certain nuclear functions in the cycling basal cells, but would move into the nucleus and start affecting gene expression and other activities only with the disappearance of the CDKs in the post-proliferative suprabasal cells.

According to the most recent information,[202] it seems, as first suggested by Whitfield,[1] that PTHrP controls keratinocyte proliferation and differentiation in *mature*

mouse skin in the same way it controls endochondral bone formation and drives the growth and branching of mammary gland ducts.[203,204] Prehypertrophic chondrocytes make and secrete a paracrine factor called Indian hedgehog, which stimulates prearticular perichondrial cells to make and secrete paracrine PTHrP, which feeds back onto the receptor-bearing prehypertrophic cells and by promoting the expression of the antiapoptogenic Bcl-2 protector protein restrains their further terminal differentiation into the hypertrophic cells of the calcifying and vascular invasion zones that die through the action of the apoptogenic Bax protein.[205] A failure to make PTHrP results in a failure to express the Bcl-2 protector and consequently premature terminal differentiation and Bax protein-promoted apoptosis, which results in reduced resting and proliferative zones and a deformed rib cage, shortened mandible, and fore-shortened limbs.[202,205] In PTHrP-null mice (which avoid a lethal rib cage failure because they carry a procollagen II-PTHrP transgene, which is specifically expressed in chondrocytes but not keratinocytes), there is premature diffpoptosis that produces a thin and hyperkeratotic skin without a drop in the fraction of cycling basal cells.[202] In other words, the transit time from basal cell to corneocyte is accelerated, which depletes the populations of cells in the intermediate stages of differentiation and thus the thickness of the skin. In these animals, K1 keratin expression, which is normally limited strictly to the spinous (suprabasal) layer, is initiated in some basal cells and loricrin is expressed in the upper spinous layer as well as in the granular and horny layers.[202] By contrast in mice in which keratinocyte PTHrP is selectively overexpressed in basal cells because of the activity of a K14-PTHrP transgene (for the proliferation-related keratin 14), the fraction of cycling cells is doubled (presumably not because of the protein's intrakine nuclear functions in these cycling cells), basal cells are enlarged, the basal cell layer is expanded, and differentiation is held back, as indicated by the expression of K14 even in the lower spinous layer and a displacement of K1- and loricrin-expressing cells to higher suprabasal layers.[202] Because of this the PTHrP-overexpressing skin is much thicker with marked acanthosis and papillomatosis.[202] In summary, PTHrP maintains epidermal thickness (and the complement of hair follicles and sebaceous glands) by appropriately balancing proliferation and differentiation by delaying terminal apoptosis just as it does in cartilage.

PTHrP-driven feedback circuits between keratinocytes and dermal cells are the actual balancers of keratinocyte proliferation and differentiation. Dermal fibroblasts express the conventional, adenylyl cyclase-activating type I PTH/PTHrP receptors, and the PTHrP coming down from the spinous cells stimulates the fibroblasts to proliferate and make fibronectin, an important integrin-binding (hence ILK-stimulating) component of the basal lamina.[177,206,207] Human and rat dermal fibroblasts make a soluble paracrine factor(s) that stimulates keratinocytes to produce PTHrP.[208] Therefore, the PTHrP fragments trickling down from the spinous layer stimulate the dermal fibroblasts to make and secrete growth factors such as KGF, which promotes basal cell proliferation by inducing them to make autocrine TGF-α while at the same time keeping the spinous cells from initiating the final, apoptotic stage of diffpoptosis until they have collected enough of the different components needed to become proper corneocytes possibly by promoting the expression of the Bcl-2 protector as it does in the prehypertrophic cartilage cells (Fig. 3.1).[103,139,177,205–209] Adenylyl cyclase-stimulating N-terminal PTHrP fragments also stimulate the dermal fibroblasts to make IGF-I, which the keratinocytes do *not* make but which provides "survival signals" and promotes the $G_1$ buildup in the basal cells as well

as promotes extracellular matrix synthesis (Fig. 3.1).[210] Clearly, a flood of autocrine/paracrine factors produced in response to overexpressed PTHrP would thicken the epidermis by increasing the flow of cells into the spinous layer and then delaying their differentiation and death, while the lack of KGF and IGF-I in a PTHrP-null skin would thin the skin through the accelerated diffpoptosis of the cells flowing normally into the spinous layer.

This PTHrP-mediated interaction between epithelial and mesenchymal stromal cells is not restricted to skin. PTHrP made by mammary ductal epithelial cells targets receptor-bearing stromal cells, which, when stimulated by the PTHrP, drive mammary ductal growth and morphogenesis via the same factors such as KGF and IGF-I that regulate keratinocyte proliferation and diffpoptosis.[204]

Another major driver of the diffpoptosis program is $1\alpha,25(OH)_2$ vitamin $D_3$. It operates through a nuclear receptor that binds to the so-called VDRE (vitamin D-responsive element) in the promoters of its target genes. However, it also activates surface receptors, which, like the PTHrP receptors, stimulate a PLC-β1-induced $Ca^{2+}$/PKCs cascade in keratinocytes and other cells.[211–217] In cultured keratinocytes, $1\alpha,25(OH)_2$ vitamin $D_3$ downregulates the expression of the EGF receptor and c-*myc* and can therefore stop proliferation.[218] But it also enhances the $Ca^{2+}$-mediated expression of involucrin, transglutaminase, and the formation of the cornified envelope by stimulating the expression of the CaR gene and the genes encoding PLCs such as PLCs-β2, -γ1, and -δ1 but particularly PLC-β1.[218] Indeed, $1\alpha,25(OH)_2$ vitamin $D_3$'s main mission is to keep the evolving keratinocyte highly responsive to the $Ca^{2+}$ gradient.[219] Of course, the steroid would also increase the sensitivity of the cells to the various possible PTHrP fragments, all of which activate PLC-β1.

What does $1\alpha,25(OH)_2$ vitamin $D_3$ do in the real world of the skin? It appears that there is a paracrine system in the skin in which $1\alpha,25(OH)_2$ vitamin $D_3$ is made and secreted by less differentiated cells, but it is the more differentiated cells that have the VDR mRNA and VDR.[220] It would seem reasonable to expect that signals from the receptors for the autocrine/paracrine PTHrP produced by the spinous cells could stimulate $1\alpha,25(OH)_2$ vitamin $D_3$ synthesis as they do in proximal rat kidney cells.[218,221] The $1\alpha,25(OH)_2$ vitamin $D_3$ could then, for example, directly stimulate the expression of VDR-responsive genes and CaR expression in the overlying granular cells.

## *From spinous cell to granular cell: CaRs and upward to the head of the $Ca^{2+}$ gradient*

When the cell has acquired a granular cell transcriptome and has reached the granular layer it hits a wall of $Ca^{2+}$ — the external $Ca^{2+}$ concentration triples (Fig. 3.1).[71] By now, it has loaded its surface with CaRs to detect and respond to this big jump in the external $Ca^{2+}$ concentration.[80–82,222–230] Functional CaRs are needed to trigger the final stages of diffpoptosis in the granular layer. Without them, there is a reduced expression of loricrin and profilaggrin, abnormal granule morphology, defective lamellar body secretion, and a disorganized horny layer.[223,224]

The human keratinocyte's CaR appears to be the same as the parathyroid gland chief cells' low-affinity CaR, which monitors the blood $Ca^{2+}$ concentration and triggers PTH secretion if the $Ca^{2+}$ concentration should fall below a critical level and reduces PTH

secretion if the $Ca^{2+}$ concentration should rise above a critical level.[80,81] The CaR must have a low affinity for $Ca^{2+}$ to be able to detect and respond to changes in the extracellular $Ca^{2+}$ concentration because if it had the high affinity of an ultrasensitive sensor such as intracellular CaM ($K_d \approx 10^{-6}$ M) it would be permanently activated by the millimolar levels of extracellular $Ca^{2+}$. The CaR is made up of two different peptide structures, the N-terminal one of which has come from the bacteria and the other of which has come from the eukaryotes. The large, extracellular, N-terminal $Ca^{2+}$-trapping domain is descended from the very ancient family of bacterial periplasmic ion and nutrient detectors that grab ions (like a Venus flytrap grabs flies) and then appropriately steer the cell's swimming engines toward or away from the target, while the part of the molecule with its 7-transmembrane α-helices come from the huge family of thousands of eukaryotic G-protein-coupled serpentine receptors (to which the PTH/PTHrP receptors also belong albeit to a different clan from the CaR's clan).[80,231–233a] The cation-binding ability of the protein's extracellular N-terminal domain resides in several regions rich in negatively charged amino acids.[80,81] The human keratinocyte's CaR, like the parathyroid cell's CaR, is really a polyvalent cation receptor that responds to $Ba^{2+}$ and $Sr^{2+}$ as well as to $Ca^{2+}$, but in practice in the body it is effectively a selective $Ca^{2+}$ sensor.[223] It triggers the release of $Ca^{2+}$ from internal stores as well as a prolonged flow of $Ca^{2+}$ into the cells by opening non-specific cation channels in the cell membrane known as SOCs (stores-operated channels) or CRACs ($Ca^{2+}$- release-activated channels).[234] It is probably coupled to phospholipase-Cγ1, which breaks PtdIns(4,5)$P_2$ down into Ins(1,4,5)$P_3$ that releases $Ca^{2+}$ from internal stores and diacylglycerols which stimulate PKCs.[225,226] The set point of the BALB/MK keratinocyte's cation receptor for responding to $Ca^{2+}$ appears to be about 0.7 mM.[227] Raising the external $Ca^{2+}$ concentration from 0.05 to 1.8 mM triggers a $Ca^{2+}$ transient in these cells that peaks 3 min later and a burst of membrane-associated PKCs activity (from diacylglycerol-induced activation of PKCs already attached to the cell membrane and a $Ca^{2+}$-induced translocation of PKCs from the cytosol to the diacylglyerol-generating/activation sites in the membrane) that peaks 7 min *after* $Ca^{2+}$.[208] This long delay between the $Ca^{2+}$ peak and the peak PKCs activity makes the signal from the CaR rather special. The expected response to such a phospholipase-C-coupled receptor would consist of simultaneous or nearly simultaneous Ins(1,4,5)$P_3$-induced surges of internal $Ca^{2+}$ and diacylglycerol-stimulated membrane-associated PKCs activity and the phosphorylation by these PKCs of the 85-kDa MARCKS (Myristylated Alanine-Rich C-Kinase Substrate) protein bound electrostatically to patches of PtdIns(4,5)$P_2$ that it has organized in the membrane near PLC-γ1, but the relative slowness of the PKCs' response to activation of the CaR gives the $Ca^{2+}$·CaM complexes produced by the surging $Ca^{2+}$ in time to reach and block the MARCKS proteins' phosphorylation site-domains (and probably those of some other signal-mediating proteins) before the activated PKCs can get to them.[227,228] The functional significance of this striking blockage of MARCKS phosphorylation and possibly other major signaling components is unknown, but it is probably a very important characteristic of CaR signaling. The CaR-induced $Ca^{2+}$·CaM triggers a wide-ranging cascade of reactions mediated by CaMKs II and IV, and the protein-serine/threonine/tyrosine phosphatase calcineurin.[1,235,236] Activated CaMKs such as CaMKIV mimic cyclic AMP by phosphorylating and activating the CREB (cyclic AMP-responsive element-binding) protein transcription factor that stimulates the expression of those cyclic AMP genes that are configurationally accessible for

transcription in granular keratinocytes.[1,237] The membrane becomes a very busy place because the $Ca^{2+}$ surge also stimulates the translocation of phospholipase A2 from the cytosol to plasma membrane where it hydrolyzes membrane phospholipids and releases arachidonic acid, the precursor of prostaglandins and other eicosanoids.[238,239] It is important to note as our cell approaches the granular stage that in one study 91% of the total $PLA_2$ activity in human skin was found in the cells of the granular and horny layers,[240] which suggests a special role for $PLA_2$-generated eicosanoids in the later stages of diffpoptosis.

The CaR of murine, but not human, keratinocytes is also specifically coupled to an as yet unknown protein tyrosine kinase that phosphorylates a 62-kDa protein associated with the Ras-GTPase-activating protein when the receptor is activated by $Ca^{2+}$, nickel, or cobalt.[230,241] This protein kinase is activated within 2 min by the receptor protein, but not by an internal $Ca^{2+}$ surge, because $Ca^{2+}$ ionophores such as the A23187 and X537A that raise the intracellular $Ca^{2+}$ content do not stimulate the protein kinase, while $Ni^{2+}$, which activates the CaR, stimulates the kinase without significantly raising the internal $Ca^{2+}$ level. The $Ca^{2+}$ surge triggered by the murine CaR activates a protein tyrosine phosphatase (perhaps the protein-serine/threonine/tyrosine protein phosphatase calcineurin?) that activates Fyn, a Src-family protein tyrosine kinase, by a process involving dephosphorylation of the enzyme's $Tyr^{531}$ residue, which causes a configurational change that exposes the enzyme's catalytic site. This surge of protein kinase activity starts 1 h after $Ca^{2+}$ addition and lasts up to 24 h.[1,230]

The basic diffpoptotic plan is first to stop the cell from cycling, then to make keratin cables and plug some of them into desmosomes to link the cell strongly and flexibly to its neighbors, then to load the cell with keratins and the several things needed to make a cornified envelope, and finally to activate the mechanism that will use these things to convert the cell into a hollowed-out corneocyte packed with keratin macrofilaments attached to the inner surface of its lipid-coated horny shell.

On the threshold of the granular layer, the spinous cell has stopped expressing the CLED protein that was triggered by the detachment signal.[139a] It has also stopped displaying large numbers of KGF receptors and is thus no longer protected by the terminal diffpoptosis-suppressing action of KGF from the PTHrP-stimulated dermal fibroblasts: it is now ready to advance to the final, apoptotic part of diffpoptosis.[139] It is much flatter; packed with a network of heavy keratins; riveted strongly to its neighbors by desmosomes; accumulating on its plasma membrane some cornified envelope precursors such as involucrin; still filling up with lipid lamellar bodies; and already loaded with the inactive envelope cross-linking enzyme, $TG_K$, which, with its fluctuating numbers of myristoyl and palmitoyl adducts, is cycling on and off the cell membrane waiting for the signal to start working.[76,94,154,158–162,242]

What turned on the genes coding for these components in the cell before it reached the top of the spinous layer? Experiments with cultured keratinocytes have shown that a 0.03→0.12 mM $Ca^{2+}$ shock shuts down keratins 5 and 14 production and activates the $Ca^{2+}$-responsive element in the keratin 1 gene's regulatory region, which is followed by the stimulation of the keratin 10 production by the accumulating type 1 molecules (Fig. 3.3).[1,84,85,90–92] A 0.3→1.2 mM $Ca^{2+}$ shock shuts down keratin 1 and 10 gene expression and protein production and stimulates the production of "granular cell/corneocyte" components such as filaggrin, involucrin, loricrin, tonofilaments, and $TG_K$

(Fig. 3.3).[1,84,85,90–92] Exposure to the PKCs activator, 12-*O*-tetradecanoyl phorbol-13 acetate, promotes the appearance of granular cell-specific gene-transactivators, shuts down the production of keratins 1 and 10 mRNAs and proteins while simultaneously stimulating the expression of filaggrin and loricrin mRNAs and proteins by activating the "classical" $Ca^{2+}$-dependent PKCα as well as transiently stimulating the production of $TG_K$ mRNA in human cells by activating the "novel" $Ca^{2+}$-independent PKC-η.[85,91,243,244]

One difficulty with labeling components such as involucrin and the cross-linking $TG_K$ as granular stage-specific in the cell culture experiments is that in the real world of the skin involucrin accumulates in the upper *spinous* layer and $TG_K$ *immuno*reactivity *peaks* at the top of the *spinous* layer.[159,160] Another difficulty is the number of different stimulators of $Ca^{2+}$ surges and bursts of membrane-associated PKCs that are known to stimulate the differentiation of cultured human and murine keratinocytes. First, there is the $Ca^{2+}$ surge that occurs when the cell cuts off the flow of β1-integrin signals and detaches from the basement membrane (or culture dish). Then, there is the $Ca^{2+}$/PKCs signal from the spinous cells' conventional PTH/PTHrP and TRSAW receptors. Finally, there are stronger and longer $Ca^{2+}$/PKC signals from the CaRs to granular cell-specific nuclear transcription factor complexes when the external $Ca^{2+}$ concentration triples upon entry of the cell into the granular layer and continues rising into the lower horny layer (Fig. 3.1).[222]

How can a tandem sequence of what seem to be the same $Ca^{2+}$/PKCs signals stimulate the expression of the different sets of genes of keratinocyte diffpoptosis? While the $Ca^{2+}$ signals seem to be the same when they are given at different points in the program, they are in fact not the same. As we learned in the previous chapter, $Ca^{2+}$ released from storage vesicles or flowing into the cell through membrane channels is bound and sequestered very fast and thus cannot diffuse very far from the point of release or portal of entry. Therefore, as the cell differentiates, the $Ca^{2+}$ signals in the forms of quarks, blips, sparks, spikes, repetitive spikes, and (or) waves are differently sited and configured according to the location of their source and their frequency (FM) and amplitude (AM), and they are sent along changing selective intracellular pathways and tunnels toward shifting sets of targets.[245]

When the cell reaches the granular layer, it switches over to another set of transcription factors. For example, it starts accumulating C/EBPα.[140] Among the most important of the many transcription factors involved in diffpoptosis are those that make up the AP1 complexes, which are activated to bind target promoter sequences of DNA by being phosphorylated by PKCs. These complexes are heterodimers of Fos-family proteins (which cannot by themselves bind to AP1 motifs in DNA) and Jun-family proteins (homodimers of which can bind to AP1 motifs but with a much lower affinity than Fos/Jun heterodimers).[246] The genes encoding these components are differently expressed in response to bursts of changing PKC isoform activities as the reconfiguring cells move from layer to layer. In proliferating cultured mouse basal keratinocytes, the DNA-binding activity of AP1 complexes is very low, but is increased by the bursts of PKCs α, δ, and ε (but not η) activity triggered directly (PKC-α) or indirectly (PKCs δ and ε) by raising the $Ca^{2+}$ concentration in the medium from the proliferation-supporting 0.05 mM to the differentiation-triggering 0.12 mM.[247,248] The AP1 complexes in the $Ca^{2+}$-stimulated cells consist of Fra-1 or Fra-2 and c-Jun, Jun B, or Jun D, but not c-Fos.[247,248] The PKCs activity also stimulate the expression of the *fra*-2, *jun* B, and *jun* D

genes.[247] In the real world of the mouse skin, it appears that AP1 complexes containing Fra-1 and Jun D predominate in spinous cells, while AP1 complexes containing Fra-2 and Jun B predominate in granular cells.[247] Increasing the $Ca^{2+}$ concentration in the growth medium of human keratinocytes from 0.03 to 1.2 mM also increases the expression and nuclear levels of Fos B, Fra 2, and Jun B.[249] In human skin, the c-*fos* gene is not expressed in basal cells but is expressed in the upper spinous and granular layers and lower granular layer; the *fra*-1 gene is also not expressed in basal cells, but it is switched on when the cells reach the first or second spinous layer and is turned off again when the cells reach the upper granular layer; *fos* B and *fra*-2 are expressed in basal cells, but are increasingly expressed when the cells are in the lower and upper spinous layer, respectively, and are then turned off by the time the cells reach the granular layer; *jun* is expressed *only* by granular cells; and *jun* B and D are expressed by cells in all of the layers (Fig. 3.5).[250]

Whether or not, or how, the genes for involucrin, keratins, loricrin, profilaggrin, and transglutaminase $TG_K$, which have AP1-binding motifs in their promoters,[249,251–254] respond to the $Ca^{2+}$ and PKCs signals triggered by detachment from the basal lamina, activation of PTHrP receptors or activation of the CaRs depends on the responsiveness of their promoters to specific AP1 complexes. For example, PKCs-phosphorylated AP1 heterodimers containing Fra-1 and Jun B or Jun D *stimulate* keratin 1 and involucrin

**Fig. 3.5.** The same $Ca^{2+}$ and PKCs (particularly PKC-α) signals from integrin detachment, PTHrP, and cation receptors stimulate or inhibit the expression of different sets of genes at different stages of the diffpoptosis program. The different responses discussed in the text to the superficially same signal are due in large part to stage-specific differences in the expression of different components of AP1 transcription complexes that are phosphorylated and activated by PKCs. Here, we see the shifts in AP1 components and their intracellular locations (superscript c indicates cycoplasmic location, while superscript n indicates nuclear location) that occur during human keratinocyte diffpoptosis. The point in the progression from basal cell to granular cell when a component peaks is indicated in bold color-coded letters (e.g., **Fos $B^n$** or **$Jun^{nc}$**). The dramatic consequences of such shifts can be illustrated with one example. In spinous cells, PKCs-phosphorylated AP1 complexes containing Fra-1, Jun B, and Jun D stimulate the expression of keratin 1 and involucrin genes and at the same time inhibit profilaggrin gene expression. However, when c-Jun appears and peaks in granular cells, it displaces Jun B and the resulting new AP1 complexes shut down the keratin and involucrin genes and turn on the profilaggrin and loricrin genes.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Granular ↑ | $Fos^{nc}$<br>$Fos^{nc}$<br>$Fos^{nc}$ | | $Fra\text{-}1^{c}$<br>$Fra\text{-}1^{c}$ | | **$Jun^{nc}$** | $Jun\ B^{c}$<br>$Jun\ B^{c}$<br>$Jun\ B^{c}$ | $Jun\ D^{n}$<br>$Jun\ D^{n}$<br>$Jun\ D^{n}$ |
| Spinous ↑ | $Fos^{nc}$ | $Fos\ B^{n}$<br>**$Fos\ B^{n}$**<br>$Fos\ B^{n}$<br>$Fos\ B^{n}$ | $Fra\text{-}1^{c}$<br>$Fra\text{-}1^{c}$ | **$Fra\text{-}2^{nc}$**<br>$Fra\text{-}2^{nc}$<br>$Fra\text{-}2^{nc}$<br>$Fra\text{-}2^{nc}$ | | $Jun\ B^{c}$<br>$Jun\ B^{c}$<br>$Jun\ B^{c}$<br>$Jun\ B^{c}$ | $Jun\ D^{n}$<br>$Jun\ D^{n}$<br>$Jun\ D^{n}$<br>$Jun\ D^{n}$ |
| Basal ↑ | | $Fos\ B^{n}$ | | $Fra\text{-}2^{nc}$ | | $Jun\ B^{c}$ | **$Jun\ D^{n}$** |

expression in spinous cells,[254,255] but they *repress* profilaggrin gene expression.[252] However, the appearance and buildup of c-Jun in granular cells would displace Jun B from AP1 sites and thus suppress keratin and involucrin gene expression and stimulate profilaggrin and loricrin expression.[251,252]

Judging from the results of experiments with cultured keratinocytes, our cell probably has a larger complement of various PKCs and greater PKC-stimulated AP1 DNA-binding activity when it reaches the top of the spinous layer than it had when it left the basement membrane.[247,256–258] The strengthening PKCs signals from PTHrP receptors and now the $Ca^{2+}$-activated polyvalent cation receptors change the composition of AP1 complexes, which shut down the expression of the genes for keratins 1 and 10 (but not the *production* of these proteins), and destabilize the keratin 1 mRNA transcripts.[259] However, as the cell crosses into the granular layer it stops making and processing PTHrP and installing gap junctions (Fig. 3.1).[96,99,100,172,173,176] It continues accumulating the lipid lamellar bodies.[104]

The granular cell's mission is to switch on the rest of the EDC genes in the chromosome 1q21-22 cluster (Fig. 3.4) to make the remaining cornified envelope components. The cell then accumulates the cytoplasmic keratohyalin granules, which give it its name.[104,260] There are three kinds of granule: the P-F-granule, which contains highly phosphorylated profilaggrin; the L-granule, which contains loricrin; and the composite granule, which contains profilaggrin surrounded by loricrin.[260]

The 350-kDa profilaggrin molecule has multiple filaggrin repeats linked by short peptides, a $Ca^{2+}$-binding S-100 protein-like N-terminal domain, and several phosphorylation sites.[168–170,260] It seems likely that the CaR-triggered $Ca^{2+}$ and PKCs surges and the granular cell-specific Jen transcription factor[261] work together to stimulate the expression of the profilaggrin gene. Then, $Ca^{2+}$ and a group of $Ca^{2+}$-independent protein serine/threonine kinases (e.g., casein kinase II) collaborate to pack the highly phosphorylated, insoluble protein into the P-F-granules, which are deposited at points of intersection of keratin filament bundles and progressively enlarge as the cell rises to the top of the granular layer.[104,260] The $Ca^{2+}$ surges triggered by the granular cell's CaRs also strongly stimulate the expression of loricrin (from Latin: *lorica*, shell or cover), a very glycine-rich protein which has tandem Gly–Ser repeats separated by glutamine-rich sequences and flanked by glutamine-lysine-rich motifs which will be used by $TG_K$, and the later-appearing loricrin-selective $TG_E$, to cross-link the protein into the cornified envelope.[159,160,262] The glycine–serine regions form inverted "Ω-loops," which will be used to dock and anchor keratin macrofilaments to the inner surface of the future horny envelope.[94] Like profilaggrin, loricrin is rendered insoluble by phosphorylation and self-association through disulfide bonding and sequestered in the L-granules.[260] The storage of loricrin and profilaggrin as inactive, insoluble precursors in granules prevents premature and aberrant cornification and keratin filament aggregation. Loricrin as well as the other components of the keratohyalin granules must be shielded from the massive proteolysis by lysosomal cathepsins B and H during the granular→corneal transition. The shield is keratolinin (cystatin A) that began accumulating in and around the granules during the spinous stage.[94,260]

Now we must remember the dramatic, mid-granular buildup of intercellular $Ca^{2+}$ and the increase in the intracellular $Ca^{2+}$ concentration that occurs when cells reach the top of the granular layer and the head of the $Ca^{2+}$ gradient and cross into the horny layer

(Figs. 3.1 and 3.2).[74,75] This intracellular $Ca^{2+}$ surge is probably due to the increasing signaling by the CaRs, responding to the external $Ca^{2+}$ buildup (Figs. 3.1 and 3.2). The cell now enters a transitional state in which it must start processing the accumulated keratohyalin granule components to make the horny envelope. The keratohyalin granules start thinning and their contents disperse throughout the cytoplasm.[104] Profilaggrin is processed in two stages. First, a $Ca^{2+}$-independent chymotrypsin-like protease chops it into oligomers, which are then chopped into filaggrin monomers by a $Ca^{2+}$-activated protease.[260] The filaggrin monomers then associate with the keratin filaments to form a temporary scaffolding to orient the filaments until they are locked in place by disulfide bonds. The components of the loricrin aggregates are released (by the reduction of their disulfide linkages) for cornified envelope formation and for docking keratin macrofilaments to their "Ω-loops."[94,260] As various components have been moving to the cell surface to join involucrin and the waiting, but still inactive, $TG_K$ and $TG_E$, they form a distinct growing band beneath the soon-to-disintegrate plasma membrane.[160]

## *The final push to cornification and oblivion*

The corneocyte is created by a modified form of apoptosis in the component-loaded transitional cell. A feature of this apoptoid process is the use of the specific membrane-associated transglutaminases, $TG_K$ and $TG_E$, instead of the cytoplasmic "tissue transglutaminase," $TG_C$, that is used in the non-specialized or general apoptotic mechanism designed simply to get rid of cells as quickly as possible without damaging neighboring cells.[57,94] But the keratinocyte must not be quickly destroyed: its apoptotic transformation to corneocyte is the crowning event of diffpoptosis. The corneocyte must stay around for a while as a part of the body's horny covering.

Apoptosis was invented to enable cells to kill themselves without damaging or killing their normal neighbors by releasing a murderous pack of endonucleases, lipases, phosphatases, and proteases such as the caspase cysteine proteases (ICE [interleukin-1β converting enzyme]-like proteases) during their death throes.[135,263,264] The keratinoctye-specific $TG_K$ and the loricrin-specific $TG_E$ transglutaminases are special guest players in keratinocyte diffpoptosis, which, from their perches on the cell periphery, grab *any* passing large or small proteins with available glutamine and lysine residues and cross-link them with isopeptide bonds into an impervious horny shell while the cell's plasma membrane, nucleus, chromosomes, and other organelles are being shredded by a ravenous horde of hydrolytic enzymes.[1,94,159,160] This part of the diffpoptosis program has been ingeniously adapted to make skin and scales by first having the keratinocyte use $Ca^{2+}$ surges and bursts of various PKCs activities to accumulate polyamines and a pool of large and small glutamine- and lysine-rich proteins that have been specifically designed for making a cornified envelope.[94]

When the granular-transitional cells have made enough of the things needed to become corneocytes and the external $Ca^{2+}$ concentration is high enough, the terminal transition is fast. The death machinery swings into action, probably in response to signals from the CaRs that activate a starter protease such as the SCCE (stratum corneum chymotrypic enzyme) or that stimulate the secretion of an autocrine starter protease or that stimulate a starter caspase (ICE-family protease).[57,133,264–266] It seems likely that $Ca^{2+}$ cycling into and out of the mitochondria induced by the intense $Ca^{2+}$ signaling in these

cells opens megachannels in the mitochondrial inner membranes through which the apoptogenic caspase proteases can pass into the cytoplasm and reach key target sites.

The inactive TGs that have been patiently perched on the inner surface of the CaR-armed cell since their appearance and accumulation during the spinous stage now come alive when the CaR signaling peaks at the $Ca^{2+}$-rich interface of the granular and horny layers (Figs. 3.1 and 3.2). $Ca^{2+}$ activates the full-length 106-kDa $TG_K$ on the cell membrane by stimulating its partial proteolytic processing at specific sites by a CANP(s) ($Ca^{2+}$-activated neutral protease[s] or calpain[s]) into highly active complexes of 10-, 33-, and 67-kDa fragments that are anchored to the membrane by the 10-kDa fragments.[158,159,267–269] The intense $Ca^{2+}$ signaling also activates the loricrin-specific $TG_E$. The active TG complexes cross-link cornifin, involucrin, loricrin, pancornulins, and some other proteins (e.g., 36-kDa annexin 1 and a 61-kDa dimer of annexin 1 [driven onto the membrane by $Ca^{2+}$] or another protein) into the tough, 7–15-nm horny envelope subjacent to the cell membrane with involucrin on the outer surface of the envelope and $TG_E$ selectively cross-linking loricrin into the inner surface of the envelope.[94,159,160] Also somehow involved in regulating this final stage of diffpoptosis is the recently discovered $Ca^{2+}$-binding CLSP (Calmodulin-Like Skin Protein), which, unlike CaM, binds to TGs.[269a]

As this is happening the keratin-associated filaggrin monomers, which have been serving as a temporary scaffolding for the keratin network, dissociate from the keratin filaments when their positively charged arginine residues are deaminated to citrulline and degraded to free amino acids.[260] The liberated keratin macrofilaments are now attached to the Ω-loops hanging down from the loricrin on the inner surface of the horny envelope.[94] The amino acids of the dissociated, degraded filaggrin are hydrophilic and act as corneocyte moisturizers by binding water.

The $Ca^{2+}$ surges also generate $Ca^{2+}$·CaM complexes which activate the $Ca^{2+}$-ATPase pumps in the doomed plasma membrane (Fig. 3.2).[1] While activating the TGs, the $Ca^{2+}$-activated CANPs may also help switch on the $Ca^{2+}$ pumps by binding via their calmodulin-like domains to the pumps' calmodulin-binding regulatory sites and then activating the pumps by limited proteolysis.[270] This double action makes sure that once it has done its job most of the exchangeable $Ca^{2+}$ is pumped back into intercellular space to prevent a steady leakage of $Ca^{2+}$ from the body through the skin and in the process maintain the head of the transepidermal $Ca^{2+}$ gradient (Fig. 3.2).

During its progression from the spinous stage, the cell has been accumulating lamellar bodies, which may ultimately occupy as much as 10% of the cell's cytoplasmic volume.[76] When the cell arrives at the top of the granular layer, it reconfigures itself into a very special kind of secretory cell.[271] At the border of the horny layer, the lamellar bodies that were formed in and then emerged from the tubular-reticular membrane system of the trans-Golgi network, collected in aggregates and linear arrays in deep invaginations or pockets of the apical surfaces of the cell from which they can easily reach the cell surface.[271] Without these special pathways, the lamellar bodies would get trapped in the mature granular cell's maze of surface keratin filaments, keratohyalin granules, and growing patches of horny envelope. Nested in the invaginations, they are poised for rapid exocytosis. Now, under the normal circumstances of an intact permeability barrier and the head of the $Ca^{2+}$ gradient at the interface between the granular and corneum layers, the cell slowly releases the contents of its lamellar bodies into the intercellular space at

the interface of the granular and horny layers. The "accordioned" lipid membranes dumped out of the lamellar bodies uncurl, join end-to-end, and pile up into multilaminar sheets that envelop the cells in the lower two horny layers.[76] The fusion of the envelopes of large numbers of lamellar bodies with the granular cell's membrane greatly increases the cell's surface area : volume ratio as it moves up into the horny corneum.[76] The released lipids are then converted into more apolar ceramides, cholesterol, and free fatty acids by acid-activated hydrolases that were also released from the lamellar bodies.[76] These external layers of processed apolar lipids replace the cornified cells' disintegrated plasma membranes, and the ceramides are covalently linked to glutamine residues of the involucrin on the surface of the cornified envelope to form a lucent hydroxyceramide layer on the outer surface of the envelope.[76,94,160,272–275]

The product of these events is the all-important permeability barrier the invention of which was a sine qua non for the emergence of vertebrates onto dry land.[73,76] $Ca^{2+}$ controls the establishment of the permeability barrier. Removing the barrier (e.g., by repeated tape stripping to remove the corneum) causes a transepidermal water loss and eliminates the $Ca^{2+}$ gradient. This $Ca^{2+}$ depletion and silencing of the upper granular cells' CaRs causes a massive release of lamellar bodies and increases the expression of the genes encoding various enzymes for lipid synthesis (acetyl-CoA carboxylase, farnesyl pyrophosphate synthase, fatty acid synthase, HMG-CoA reductase, serine palmitoyl transferase, squalene synthase) to restore both the lipid barrier and the $Ca^{2+}$ gradient.[73,76] It also triggers the expression and release of a specific group of cytokines (IL-1α, IL-8, IL-10, intercellular adhesion molecule-1 (ICAM-1), interferon-γ, TGF-α, TGF-βs, TNF-α) that stimulate an inflammatory response, lipid synthesis by suprabasal cells, and the proliferation of basal cells.[76,276–280] Particularly important among these is TNF-α, which can stimulate its own expression as well as those of ICAM-1, IL-8, the all-important TGF-α which appears within 1 h and stimulates keratinocyte proliferation, motility, and matrix formation.[277] It seems that TNF-α is located mainly in the granular cells, which, however, do not express their TNF-α gene. But they must have expressed the gene at some earlier stage (probably when they were KGF-stimulated cycling basal cells) and accumulated a store of the cytokine for release in case of barrier disruption.[277] The released TNF-α then spreads to underlying cells, which it stimulates to express their own TNF-α genes as well as those of the other proliferogenic factors.[277,278] Among these underlying target cells may be the dermal fibroblasts, which respond by making more KGF for increasing the basal cells' TGF-α expression and proliferation.[68] However, the proliferogenic response is soon ended (e.g., by 6 h) when the proliferation-phase factors stimulate the expression of the growth-inhibitors such as TGF-β(s) and interferon-γ.[277]

Prematurely restoring signaling from the divalent cation sensors by adding $Ca^{2+}$ (and $K^+$) to the bathing solution prevents the massive expulsion of lamellar bodies and the burst of lipid synthesis that are urgently needed to promptly restore the permeability barrier.[72,73,76] Evidently, the signals from the granular cells' CaRs do three very important things when the barrier is restored and the gradient is reestablished: signal the presence of the restored permeability barrier; switch off the mechanism that responded to the barrier disruption; and trigger the apoptotic coversion of the cells into lipid-coated corneocytes.

So far, the cells have been bonded together by cholesterol sulfate glue made by the spinous cells' cholesterol sulfotransferase and, of course, the desmosomes. Now, the glue is destroyed by cholesterol sulfatase, which the cells release separately from the

lamellar bodies. The acid-activated proteases from the lamellar bodies cut the desmosomal tethers.

Having triggered the key steps of the diffpoptosis program, $Ca^{2+}$ now switches from the constructive Dr. Jekyll to a murderous Mr. Hyde. The phosphatases, phospholipases, proteases, and nucleases such as DNAase I (liberated from its complex with G-actin by the rampaging proteases)[133] that have been unleashed by the strong $Ca^{2+}$ signals from the CaRs and the cascade of events unleashed by caspases leaking out of the disintegrating mitochondria destroy the cell's nucleus and other organelles, leaving a horny shell, purged of its $Ca^{2+}$ and packed with keratin cables anchored to loricrin Ω-loops sticking out of the shell's inner surface. The final products of diffpoptosis are horny cadavers embedded in a water-impermeable lipid, which will eventually be sloughed off into a cellular oblivion.

## Summary

The epidermis consists of stacks of upwardly rising keratinocytes with changing transcriptomes for making the things needed for them to end up as dead hard-shelled corneocytes packed with keratin macrofilaments that are sloughed off at the top and replaced at the bottom from clusters of intermittently cycling, clonogenic stem cells and a network of larger, faster cycling amplifying transit cells. The epidermis maintains a steep, upwardly increasing $Ca^{2+}$ gradient (equal and low in the basal and spinous layers, then two and three times higher in the granular and corneum layers, respectively) through which the cells must pass. As the transit amplifying cells proliferate in the low-$Ca^{2+}$ basal layer, their adhesion to the basement membrane by β1 integrins weakens with each cycle and increasing distance from the natal niche. When the adhesion-dependent, β1 integrin-mediated signaling drops below a certain level, the cell no longer makes among other things the Cdk2·cyclin A protein kinase that is needed at the end of the $G_1$ buildup to trigger and then drive DNA replication. Detachment causes the release of $Ca^{2+}$ from intracellular stores and a surge of $Ca^{2+}$ through membrane channels. The cell starts making the $p21^{Cip/WAF1}$ and $p27^{Kip1}$ inhibitors that prevent the expression of the genes for the principal DNA-replicating enzymes by inhibiting the Cdk2·cyclin E protein kinase. The $Ca^{2+}$ signal triggers the expression and production of keratins 1 and 10 as well as involucrin and SPRR1 (cornifin), the first of the future cornified envelope components, and the $TG_K$ that will collaborate with the later-appearing $TG_E$ to cross-link them and others into the cornified envelope. The downregulation of the basement membrane-binding β1 integrins upregulates the expression and display of the $Ca^{2+}$-dependent cadherins and assembly of desmosomes to spot-weld the new suprabasal cell to its neighbors and give it the characteristic spinous morphology.

Besides accumulating proliferation-incompatible heavy keratins and the first of several envelope components, the growing spinous cell starts making intracrine PTHrP fragments, which enter the nucleus either directly from the ribosomal factory or in endocytosed receptor complexes and bind to the fibrillar component of the nucleolus where it increases the production of ribosomal RNA for making various spinous-specific components. The PTHrP fragments are also paracrine/autocrine activators of surface receptors, which generate $Ca^{2+}$ surges and bursts of membrane-associated PKCs activity that could drive the spinous program, initiate the granular program, and maintain tissue

homeostasis by modulating basal cell proliferation and the production of basement membrane components and other soluble factors by dermal fibroblasts. However, the fragments also control the sizes of the spinous and granular layers and therefore the thickness of the epidermis by delaying the onset of apoptosis by stimulating the expression of the anti-apoptotic Bcl-2 protector protein.

When the cell lifts into the granular layer, it flattens with the inner surface of its plasma membrane studded with involucrin and inactive transglutaminases, stops making gap junctions and PTHrP, and downregulates keratin gene expression using newly appearing, granular cell-specific AP1 complexes and other granular cell-specific transcription factors such as C/EBPα and Jen. It uses these granular cell-specific factors to make the rest of the envelope components such as loricrin as well as the keratin intermediate filament cross-linkers trichohyalin and profilaggrin, which it stores in keratohyalin granules until the $Ca^{2+}$-triggered signaling from the CaRs reaches a critical intensity. The extracellular $Ca^{2+}$ level does not rise until the cell is halfway up the granular layer. Then, it jumps up dramatically, and the increasingly intense and prolonged signals from the cell's CaRs start driving the final stages of diffpoptosis as the cell rises into the upper granular and lower corneum layers. These signals trigger the breakdown of the keratohyalin granules and the release of loricrin and filaggrin monomers into the cytoplasm. The filaggrin monomers then form a scaffold for orienting and cross-linking the keratin 1·10 heterodimers into a dense macrofibrillar network. The cell membrane starts to disintegrate as the $Ca^{2+}$-activated, membrane-associated transglutaminases cross-link involucrin, then loricrin and other proteins and peptides into its underlying replacement, a horny envelope with loricrin on its inner surface to attach keratin macrofilaments and involucrin on its outer surface to anchor a lucent hydroxyceramide layer after the disintegration of the cell membrane. At the same time, several enzymes that have been activated, released from mitochondria, or otherwise mobilized by the $Ca^{2+}$ pulses, kill the cell by destroying the nucleus and other organelles. The $Ca^{2+}$ surges also switch on the cell's $Ca^{2+}$ pumps, which dump all of the developing corneocyte's $Ca^{2+}$ load into a large external reservoir, the head of the transepidermal $Ca^{2+}$ gradient, which provides the external $Ca^{2+}$ that will seep down and drive the diffoptosis of the upcoming granule cells. Thus, we have seen $Ca^{2+}$ in all of its roles. We have seen it as a driver of the various stages of the basal cell's growth-division cycle, then as a differentiator that triggered the key steps in the progression from detached basal cell to spinous cell, mature granular cell, and finally as an apoptogenic killer that turned on the machinery that trashed the cell's organelles and then bailed out of the cell on the pump it turned on, leaving a dead, cornified shell packed with a dense network of keratin macrofilaments.

## Cancerous keratinocytes

### *On the road to malignancy*

The proliferation of normal keratinocytes in the skin is limited to the basal layer by the need for adhesion-induced signals from β1 integrins and associated receptors for soluble growth factors to activate PtdIns-3K, ILK, PKB, the diffpoptosis-suppressing Id proteins, suppress the expression of the $p21^{Cip/WAF1}$ and $p27^{Kip1}$ cyclin-dependent protein kinase inhibitors, and keep β-catenin flowing into the nucleus to keep the cell cycle genes

and the gene for a keratinocyte-specific tumor suppressor active. When these signals stop and the integrin signals from adhesion complexes fade, the cell detaches from the basement membrane, generates a $Ca^{2+}$ surge, shuts down the Id diffpoptosis suppressors, upregulates the p21 inhibitor, shuts down its cell cycle genes, and dismantles its DNA-replicating synthesomes. A second line of defence against hyperproliferation is the restraint imposed by direct contact with neighboring normal cells that does not involve gap junctions. A third line of defence against ectopic, suprabasal proliferation is responsiveness to the higher $Ca^{2+}$ concentrations and anti-proliferation signals from the cell's CaRs in the upper layers. Thus, during the intense competition and ruthless selection leading to the hard-won dominance of a clone that has collected the "full house" of mutations needed to cross the line of no return into invasive skin cancer,[280a] mutant cells *must* appear which

- No longer need attachment to the basal lamina to make the CDKs and replication factories;
- Are not proliferatively restrained by contact with normal neighbors;
- Are not stimulated to diffpoptose by $Ca^{2+}$and signals from CaRs.

When normal cells lift off the basement membrane because of the lowering affinity and density of integrin receptors, they reduce or switch off their cell cycle genes and CDKs but retain their death machinery, which is activated near the top of the stack and the head of the $Ca^{2+}$ gradient. Things change when the carcinogenic clonal selection process leading to papillomas and squamous cell carcinomas is initiated by a carcinogen-induced mutation, most commonly a c-Ha-*ras* gene mutation that results in the production of a constitutively activated Ha-Ras protein.[281–284] (We will learn in the next chapter that it is a mutant Ki-Ras instead of a mutant Ha-Ras that usually drives colon carcinogenesis.) This protein confers an activated phenotype on the cell by enhancing PtdIns(4,5)$P_2$ breakdown and bypassing soluble growth factors and integrins by directly and continuously stimulating PI-3K, the (PtdIns(3,4,5)$P_3$) product of which stimulates components of the proliferogenic MAPK protein kinase cascade. This enhances the expression of proliferation-related genes such as c-*myc* and converts the activated keratinocyte into a mini-cytokine factory, which overproduces and constitutively secretes many proliferogenic autocine/paracrine factors among which are TGF-α, the heparin-binding amphiregulin and betacellulin growth factors that also activate EGF/TGF-α receptors,[248] bFGF, IL-1, IL-6, and PDGF, and overexpresses EGF/TGF-α receptors because of an amplified receptor gene or an inability to downregulate the receptors.[285–292] These growth factors would amplify the proliferogenic drive of the activated Ha-Ras protein.

The overproduction of, or hyperresponsiveness to, TGF-α and the other EGF relatives would prevent or at least oppose diffpoptosis and may be responsible for the ability of squamous carcinoma cells to break down the basement membrane and invade the underlying dermis.[293–295] Another effect of overexpressed EGF/TGF-α receptor expression which enables invasiveness and impairs diffpoptosis is downregulation of the expression of the $Ca^{2+}$-activated, cell–cell binding E-cadherins and redistribution of the cadherin-associated α-, β-, and γ-catenins from the inner surface of the cell membrane to the cytoplasm.[296] This ensures the maintenance of a substantial pool of free β-catenin to sustain c-*myc* expression and other proliferation-related genes.

The initiated cells could shed the need for attachment-dependent integrin signals to initiate DNA replication, and thus free themselves from their proliferative restriction to the basement membrane, by continuing to express integrins in the suprabasal layers. Indeed, psoriasis-like suprabasal proliferation can be induced in mouse skin by attaching the suprabasally (spinous) activated involucrin gene promoter to the genes for α2, α5, and β1 integrin subunits.[297] They also have been found to overproduce $\alpha_6\beta_4$ integrin receptors, some of which contain α6A and α6B splice variants, even after lift-off, which is associated with malignant conversion and expansion of the proliferative compartment into the suprabasal layers.[298,299] Provided they can still organize signaling complexes away from direct contact with basal lamina, these inappropriately expressed integrins would presumably maintain the proliferogenic survival signaling that normally needs matrix adherence. Hauser et al.[88] have also found that unlike normal murine keratinocytes, which drop their cyclin A level when detached from their substrate and are consequently blocked in S phase, the tumorigenic SLC-1 keratinocytes behave as if their integrin signaling does not stop when detached from their substrate because they do not lose cyclin A and Cdk2·cyclin A and do not accumulate the $p27^{Kip1}$ CKI or stop making DNA.

However, the real liberator from the dependence on adhesion and integrin signals for proliferation is the activated Ha-Ras protein.[283] It operates by activating PtdIns-3K by binding directly to the kinase's 110-kDa catalytic component.[28] This keeps ILK and PKB active, and β-catenin flowing into the nucleus to suppress E-cadherin expression, to keep the cyclin D1 gene expressed and to keep the c-*myc* gene and its target $Cdk4^{32a}$ gene working to make the Cdk4·D1 cycle-starting CDK and to prevent detachment-triggered programmed death (anoikis).[30,31]

Another reason why neoplastic keratinocytes with their activated Ha-Ras protein do not trigger apoptosis when detached from the basal lamina is the inactivation of the $Ca^{2+}$-independent PKC-δ.[283] This kinase is inactivated by being phosphorylated by a Src-family protein kinase, which has been stimulated by the EGF/TGF-α receptors activated by the cells' constitutively produced and secreted TGF-α.[283] It seems likely that the 40-kDa fragment of nuclear 79-kDa PKC-δ may play an important role in apoptosis by binding and inactivating the DNA-dependent protein kinase that is involved in DNA repair and by the inclusion of an apoptogenic factor in this chromatin-associated complex.[300]

The key feature of cancerous keratinocytes is a disruption of $Ca^{2+}$ control mechanisms. At first, the initiated cells in mouse skin, for example, are phenotypically normal so application of a $Ca^{2+}$-ionophore, ionomycin, directly onto the skin before starting promotion reduces the yield of tumors after promotion.[301] However, $Ca^{2+}$-insensitive mutants ultimately appear during the forced march to cancer during promotion. Indeed, such $Ca^{2+}$- insensitive mutants must be the first to appear, otherwise the initiated cells would be stimulated to diffpoptose when they leave the low-$Ca^{2+}$ basal layer. Thus, the escape from the clutches of external $Ca^{2+}$ occurs in the early stages of chemical and viral (e.g., human papilloma virus 16) carcinogenesis; initiated but phenotypically semi-normal mutants with basal cell markers may respond to high external $Ca^{2+}$ by only reducing, instead of stopping, proliferation and uncoordinately initiating some, but not all, parts of the differentiation program.[302–314] Eventually, mutants appear, which do not need a small amount of external $Ca^{2+}$ to cycle and do not respond to the normally differentiation-triggering higher concentrations of the ion and may even be stimulated to proliferate by

these higher concentrations.[310] As we learned above, $Ca^{2+}$ shock causes normal keratinocytes in culture dishes to express their involucrin and transglutaminase genes, make involucrin and transglutaminase, move the new transglutaminases from the cytosol to the plasma membrane where they are eventually activated, and make cornified envelopes. Human SCC4 squamous carcinoma cells are examples of the loss of responsiveness to $Ca^{2+}$. These cancer cells produce involucrin and transglutaminase mRNAs, but raising the external $Ca^{2+}$ concentration to 1.2 mM does not increase the transcripts or stimulate their translation into protein.[303] Unfortunately, we do not yet know whether this failure is due to defective CaR signaling or some downstream defect. This loss of responsiveness to $Ca^{2+}$ plus the liberation of the production of CDK components from the need for attachment-induced integrin signaling enables the initiated cells to proliferate in the suprabasal layers. It also facilitates the selective growth of clones of initiated keratinocytes because initiated cells proliferate in a high-$Ca^{2+}$ medium to form colonies while their normal neighbors stop proliferating and differentiate. Thus, after this pivotal phenotypic shift, $Ca^{2+}$ becomes a *tumor promoter* (!) rather than a tumor preventor.

Once the carcinogenic process has been initiated in a niche-anchored basal stem cell or a transit amplifying cell,[25] the descendents of that cell may be *selectively* expanded into a visible papilloma by a PKCs activator such as TPA.[313] TPA could do this by overriding the suppression of the hyperproliferation of initiated, but not yet malignant, keratinocytes by surrounding normal cells, which can be demonstrated both in the culture dish and the skin. Initiated cells that directly contact normal cells are prevented by this contact from expanding into papillomas.[284,315–318] This suppression of the proliferation of initiated cells by contact with their normal neighbors is *not* due to a flow of suppressors from the normal cells or a dilution of stimulators in the initiated cells by transfer through gap junctions because such suppression in vitro requires a high (e.g., 1.2 mM) $Ca^{2+}$ concentration that would shut gap junctions as indicated by inhibition of intercellular transfer of the dye Lucifer Yellow.[315–318] Instead, TPA works by disrupting the proliferatively suppressing attachment of the initiated cells to their normal neighbors by the $Ca^{2+}$-configured/activated E-cadherins.[150,319] TPA induces the phosphorylation of E-cadherin subunits by PKCs in cell–cell adhesion complexes.[320,321] This causes a redistribution of E-cadherins from the cell membrane to the cytoplasm, which would uncouple the initiated cells from their suppressive normal neighbors.[320,321] This would derepress the proliferogenic mechanism because the surface display of $Ca^{2+}$-activated/interlinked E-cadherins suppresses proliferation and promotes stratification and diffpoptosis.[152] Specifically, suppressing the $Ca^{2+}$-dependent, β-catenin-sequestering function of E-cadherin along with the β-catenin-sparing Ha-Ras-activated PtdIns-3K would contribute to the desensitization of the initiated cell to external $Ca^{2+}$ and the creation of a pool of free β-catenin for stimulating c-*myc* and other proliferation-related genes.[30,31,35,283]

TPA and its relatives can also promote the expansion of initiated keratinocytes into papillomas because of the cells' radically altered response to surges of PKCs activities. TPA stimulates keratinocytes to make both TGFα and TGFβ.[289] The TGFα would stimulate proliferation, but the TGFβ overrides TGFα to cause normal cells to stop cycling and differentiate.[14,313] TGFβs stop cells cycling first by inhibiting the expression of cyclin D1, the first of the $G_1$ cyclins, which is needed to form the Cdk4-cyclin D1 protein kinases that hyperphosphorylate and inactivate the Rb-family pocket proteins and the related p107 protein that block the stimulation of key replication-related genes by the E2F

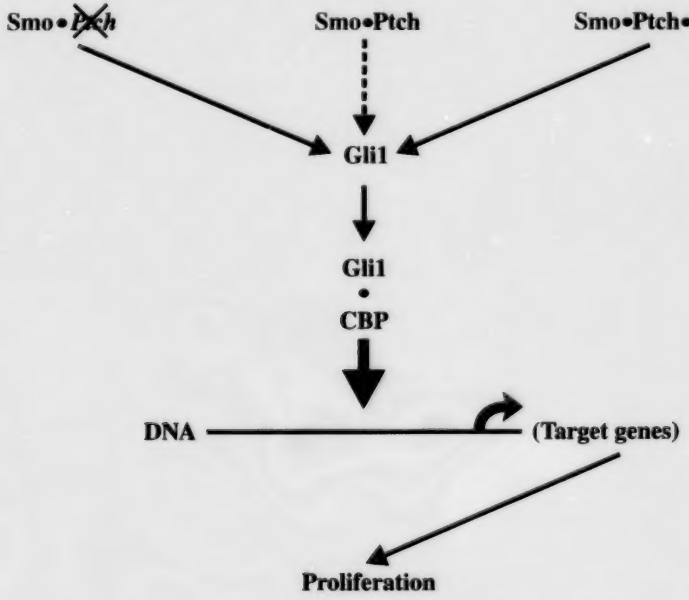
transcription factor.[123,124,322] The TGFβs also stimulate the expression of the $p15^{INK\ 4b}$ protein that selectively binds to Cdk4 protein and thus prevents CAK protein kinase (Cdk7·cyclin H) from binding to Cdk4 and activating it by phosphorylating Cdk4's $Thr^{160}$ residue. The surge of $p15^{INK\ 4b}$ also displaces the $p27^{Kip1}$ inhibitor from its complexes with Cdk4·cyclin D. The liberated $p27^{Kip1}$ can then bind to and prevent the activation of Cdk2·cyclin E, the stimulator of the principal replication genes, and Cdk2·cyclin A, the replication trigger and driver. Thus, the TGFβs stop cells cycling by blocking the $G_1$-specific protein kinases that hyperphosphorylate and thus inactivate the Rb-family pocket protein transcription-suppressors. The early initiated cells, like their normal neighbors, respond to TPA by increasing TGFβs production and increasing their already constitutive hyperproduction of TGFα, which overrides the inhibitory action of the TGFβ and stimulates the proliferation of the initiated cells. However, in mouse skin the increasingly mutation-loaded clonal descendents of the initiated keratinocyte may lose the ability to produce and (or) respond to autocrine/paracrine TGFβs on their way to becoming squamous cell carcinomas, although they might increase the production and secretion of TGFβ3 late in the carcinogenic clonal selection.[283,289,323,324] The consequence of the loss of TGFβs in mouse skin cells, or the loss of one or both parts of the active type I/type II TGFβ receptor complex, or the inactivation or loss of the TGFβ target gene, $p15^{INK\ 4b}$, the constitutive overexpression of the $G_1$-specific cyclin-dependent kinases, or the loss of the Rb-family pocket protein suppressors is that TPA, like $Ca^{2+}$, *stimulates* the proliferation of initiated cells but *stops* the proliferation of the neighboring normal cells.

Some neoplastic keratinocytes overproduce and secrete the PKCs-stimulating, hence TPA-like, PTHrP.[177,325–332] (It is relevant at this point to recall that squamous carcinoma cells display the same unconventional PTH/PTHrP receptor as normal keratinocytes that unlike the conventional receptor, does not stimulate the keratinocytes' adenylyl cyclase.[180–182]) The ability of some carcinoma cells to make and hypersecrete PTHrP depends on the ability of specific transcription factors to stimulate PTHrP gene expression and whether the cell is expressing certain mutant forms of the p53(TP53) protein (i.e., the products of p53(TP53) genes with mutants in codons 248 and 273), which can repress PTHrP gene expression.[330,332] When this PKCs-stimulating protein spills into the blood, it first stimulates bone formation, but when its concentration rises above a threshold value it stimulates $Ca^{2+}$ uptake from the glomerular filtrate by the convoluted tubule cells and demolishes bone to produce the humoral hypercalcemia of malignancy.[177,325–328] Therefore, the PKCs-activating PTHrP may be a TPA-like autocrine/paracrine proliferation stimulator for initiated, but not normal keratinocytes. Thus, the hypersecreted PTHrP, especially its intracellularly processed TRSAW-bearing fragments, might enhance tumor growth by inhibiting the proliferation and stimulating the differentiation of normal neighbors without affecting the proliferation of more advanced tumor cell clones.[188] This promoting action would apply only to squamous cell carcinomas because the PTHrP gene is not expressed in the cells of the highly invasive (but normally not metastasizing) basal carcinomas, which do not differentiate, but is strongly expressed in squamous cell carcinomas which can contain a substantial fraction of differentiated or quasi-differentiated cells.[111]

Speaking of basal cell carcinoma, the most common of the human cancers that arises in both follicular and interfollicular epidermis, it has been found that as might be expected these carcinoma cells have lost their responsiveness to $Ca^{2+}$ signals. It is the

mutational disabling or loss of a so-called "gate-keeper" gene (a gene the product of which controls the level of proliferation in a self-renewing system like the epidermis)[24] on a human basal cell's chromosome 9q that starts the clonal expansion needed to accumulate the mutations required to disable the $Ca^{2+}$ response mechanism and produce a basal cell carcinoma. This gene is called "patched" or *Ptch*.[329–339] The *Ptch* product, Ptch, is a large glycoprotein which is plugged into the cell membrane with 12 transmembrane α-helices.[330,333,340] Ptch complexes with, and inactivates, another membrane-inserted protein Smo ("smoothened"), a G-protein-coupled receptor with seven transmembrane α-helices.[330,333,340] The Ptch·Smo combination is really the modular receptor for Shh (sonic hedgehog protein), with Ptch being the ligand binder and Smo the signal generator which is inhibited by Ptch. When Shh binds to Ptch, Smo is derepressed and its signaling sends the Gli transcription factor from the cytoplasm into the nucleus where, like β-catenin, it stimulates the expression of cycle-driving genes (Fig. 3.6).[333,340]

**Fig. 3.6.** The mechanism that is the basis of basal cell carcinoma, the most frequent of the human cancers. The tumor cells have Smo ("smoothened") and Ptch ("patched") transmembrane proteins plugged into their plasma membranes. By itself, Smo would be active, but normally Smo and Ptch join to form a modular receptor for hedgehog proteins (Hh) in which Ptch silences Smo. When Hh binds to Ptch, the components separate and Smo becomes active. The Smo signaling causes the translocation of Gli1 into the nucleus where it combines with other factors to stimulate the expression of proliferation-driving genes. However, the tumor cells' Ptch genes have been mutationally disabled (symbolized as ***Ptch*** in the drawing) or lost, which unleashes Smo's constitutive proliferogenic activity. It is not yet known whether some or all *normal* basal cells use the Hh-activated Ptch·Smo mechanism to drive their cycles or whether expression or partial expression (e.g., only Smo) of the mechanism is restricted to being a first step in basal cell carcinogenesis.



Moreover, human keratinocytes retrovirally transduced with Shh expressed BMP-2, Bcl-2 protector, and other genes characteristic of basal cell carcinoma cells and produced basal cell carcinoma-like growths in nude mice.[341] Indeed, the Gli pathway may connect to the β-catenin pathway by activating the expression of the Wnt gene(s).[342] The loss or disabling of Ptch that occurs in basal cell carcinomas constitutively activates Smo and consequently Gli target genes. This results in the relentless driving of proliferation and a refusal of the cells to respond to $Ca^{2+}$ signaling to stop cycling and diffpoptose.[342a] But the question remains as to what role if any the Shh/Ptch·Smo/Gli mechanism has in normal basal cell proliferation in the mature epidermis apart from hair follicles, or does it appear in whole or in part only as the initial step in basal cell carcinogenesis?

Epidermal homeostasis is partly coordinated and maintained by the diffusion of cycle-promoters, cycle-repressors, and diffpoptosis promoters throughout the basal and suprabasal layers via gap junctions that are closed by the increasingly loud $Ca^{2+}$ signals from the CaRs when the cell hits the high extracellular $Ca^{2+}$ wall in the granular layer. The bulk of the evidence from a wide variety of epithelial and mesenchymal tumors indicates a common strong selection for mutants with disabled gap junctional intercellular communication.[102,343–357] Therefore, such cells cannot be proliferatively restrained by talking to their normal neighbors through gap junctions. However, we have already learned that the TPA-relieved, $Ca^{2+}$-requiring (*not* $Ca^{2+}$-inhibited) neighborhood suppression of the expansion of clones of early-stage initiated keratinocytes into papillomas is probably due to $Ca^{2+}$-dependent, β-catenin-sequestering, attachment of initiated cells to their normal neighbors by type E cadherins rather than gap junctions.[315–318] However, as the clonal selection of initiated keratinocytes advances toward malignancy, there is a progressive change in the structural components of gap junctions and reduction of gap junctional intercellular communication and transjunctional transmission of restraining signals from the normal cellular neighborhood.

The striking loss of gap junctional communication that can occur during the progression from papilloma to metastatic carcinoma is illustrated by the results of experiments by Klann et al.[306] Early, initiated keratinocytes were isolated from a dimethylbenz[*a*]anthracene-treated SENCAR mouse *on the basis of their resistance to proliferative inhibition by incubation in a high-$Ca^{2+}$ medium*. Early- and late-stage papilloma cells and squamous carcinoma cells were generated by a standard in vivo initiation/promotion treatment program. The intercellular transfer of microinjected Lucifer Yellow CH dye between cells in a medium containing 0.05 mM $Ca^{2+}$ dropped progressively and dramatically from a substantial 68 dye-coupled cells per injection for the early $Ca^{2+}$-resistant cells to a meager 3 dye-coupled cells for metastatic cells. Tumor promoters such as TPA profoundly affect the expression and production of the Cx (connexin) components of gap junctions of these SENCAR mouse keratinocytes.[345–347] Untreated normal cells express Cx31·1 and Cx43, but not Cx26, in vitro. However, tumor promoters switch on the Cx26 gene, transiently stimulate Cx43 gene expression, and suppress Cx31·1 gene expression. In the animal, keratinocytes also normally express Cx31·1 and Cx43, but not Cx26, but initiation by 7,12-dimethylbenz[*a*]anthracene and promotion by TPA results in Cx43 and Cx26 hyperexpression, the initiation of Cx26 hyperexpression, and repression of Cx31·1 expression by the papilloma stage.[340–347] But plasma membrane-associated Cx26 and Cx43 proteins, and presumably the gap junctions they would make, decline and disappear in the late papilloma and squamous

carcinoma stages, despite a persistent production of high levels of Cx26 and Cx43 mRNAs.[345] Thus, for some reason the cancerous keratinocyte cannot translate its abundant mRNAs into Cx proteins and install gap junctions in its plasma membrane.

On the other hand, Holden et al.[358] have found no difference in the gap junctional communication between cells from cultures of normal murine keratinocytes, papillomas, or squamous cell carcinomas, but there was a large drop in the junctional communication during the progression from squamous to spindle cell carcinoma.[358] This large drop was not due to abnormal expression of Cx43, but rather to the lack of E-cadherin expression by the spindle cell carcinoma cells, because E-cadherin mediated cell–cell contact is needed to make functional gap junctions.[358]

## The end of the road: *malignancy*

Carcinogenesis started with a rare mutation, which, instead of damaging or killing the cell, produces a cell with a radically altered response to tumor promoters and an impaired ability to diffpoptose.[314] Unless it be helped by a tumor promoter, this rare mutant cell will be suppressed and eventually vanish, or it will generate only a slowly growing clone of benign cells. But a tumor promoter will stimulate the cell and its descendants to proliferate into a papilloma. This promoter-driven clonal expansion produces the many rounds of DNA replication and opportunities for more mutations and the large number of cells needed to generate more mutation-loaded clones and eventually a malignant clone(s). While any persisting carcinogens would continue to produce mutations and thus enhance the progression to cancer, the tumor promoters that are selectively driving the expansion of the initial clone *at the expense of its normal neighbors* (which, by contrast, are being stimulated to diffpoptose rather than proliferate),[318] may also promote genetic instability and mutagenesis by triggering a shower of active oxygen radicals via the PKCs-stimulated plasma membrane-associated NAD(P)H oxidase.[359] These radicals together with $Ca^{2+}$-dependent endonuclease activity break chromosomes and thereby cause unequal chromosome exchanges with resulting gene deletions and rearrangements.[359] At first, the apoptogenic p53(TP53) safety-monitor we met in the last two chapters responds to severe chromosome damage by killing the cell to prevent the emergence of more advanced, and ultimately malignant, mutant clones.[359a] But eventually some cells may suffer a loss-of-function mutation and (or) lose their p53(TP53) gene and with it their safety-check mechanism.[284,360–365] Such cells could not accumulate enough nuclear p53(TP53) to stimulate the genes encoding the cycle-stopping, Cdk-inhibiting $p21^{WAF1/CIP1}$ and the apoptogenic Gadd45 and to organize the repair of damaged genes.[322,362,33] It might be expected that initiated, promoted, and transforming cells might also continue to produce or even over-express the anti-apoptosis Bcl-2 protector protein (which was expressed by their normal basal cell ancestor) to specifically prevent the $Ca^{2+}$-triggered apoptosis. This would further enhance their resistance to diffpoptosis especially before the loss of p53(TP53).[366] However, the evidence so far indicates that basal cell carcinomas, like normal basal cells, make Bcl-2 protector protein and do so in large amounts, but squamous cell carcinomas *do not*.[131,367] The loss of functional p53(TP53) during clonal progression removes a major obstacle to the ultimate appearance and expansion of a malignant clone, the cells of which ignore, indeed cannot respond to, $Ca^{2+}$'s iron-fisted command to stop cycling and diffpoptose.

An important requirement for the emergence of a malignant clone of invasive metastasizing cells is a failure to express E-cadherins with a resulting loss of the $Ca^{2+}$-dependent anchorage to neighboring cells by the cadherins.[368,369] This could be due, for example, to hypermethylation of CpGs around the E-cadherin gene promoter and reconfiguration of the promoter chromatin, which prevents the binding of transcription factors and thus silences the gene.[370,371] However, there may be an intermediate stage in which initiated keratinocytes still expressing E-cadherin receptors in the presence of enough $Ca^{2+}$ can (unlike normal keratinocytes) avoid anoikis and proliferate without signals from adhesion-activated β1 integrins by being included in clusters of $Ca^{2+}$-cadherin-attached, hence pseudo-adhered, cells.[372] In such clusters, the cells are close enough to each other to get strong survival- and proliferation-sustaining signals from soluble paracrine cytokines and EGF/TGF-α receptors activated by large juxtacrine TGF-α precursors sticking out of their neighbors' membranes.[69,70,372] A *premature* loss of E-cadherins would cause detached, although initiated, keratinocytes to die like detached normal cells by anoikis.[371]

Malignant conversion also needs increased AP1 transcription activity to turn on or turn up the expression of the anti-apoptosis Bcl-2 protector gene, the genes for collagenase and other proteases (e.g., stromelysin), the VEGF (vascular endothelial growth factor) gene and to turn off a novel keratinoctye tumor suppressor gene which is strongly expressed in both normal and papilloma cells.[283] These deadly converts, liberated by their mutant Ha-Ras from a dependence on adhesion-induced integrin signaling, protected from apoptosis by Bcl-2 and "immortalized" at least partially by their ever-active c-Myc stimulating the expression of their telomerase gene, can chop their way through the basement membrane with their proteinases, invade dermal blood vessels, and start new colonies in distant places where they use their VEGF to induce the sprouting of local blood vessels to nourish them.[22,35,283,373] However, these new blood vessels are not just conduits for food and oxygen. Their endothelial cells also promote the proliferation and survival of the tumor cells crowding around them by secreting paracrine growth factors such as bFGF (FGF-2) and anti-apoptosis survival factors such as IGFs.[375] However, they may have a problem making new blood vessels and producing a growing tumor because the metalloproteinases that they used to escape from the mother tumor also chop off the N-terminal parts of plasminogen and collagen XVIII to respectively release the angiostatin and endostatin fragments that block the formation of the colony's blood vessels by overriding the angiogenic actions of FGF and VEGF.[374,375]

## A farewell to the skin

We will now visit another example of a cell system where we will see $Ca^{2+}$ exercising its full range of cycle- and diffpoptosis-driving functions — the colon epithelium. But before trying to piece together what happens to a colonocyte, especially from the $Ca^{2+}$ viewpoint, as it glides up the wall of a colon crypt, let us take one final brief look at the diffpoptotic development of a keratinocyte as it rises to the top of the epidermal stack, since we shall see many similarities in the two systems. The epidermal population is maintained by the β1-integrin-primed proliferation on the basement membrane of diffpoptosis-committed transit amplifying cells, which are the descendants of niche-bound stem cells. The stratification and the stepwise differentiation of the cells started by

the interruption of β1 integrin signaling are driven by three main $Ca^{2+}$ signals. The first signal is triggered by detachment from the basal lamina and starts the production of keratins, some components of the future cornified envelope, and the expression and display of the $Ca^{2+}$-activated E-cadherin molecules that tether the cell to its neighbors rather than the basal lamina. The next signal is triggered by autocrine/paracrine factors such as PTHrP in the spinous layer to start and control the granular cell program of production of the rest of the corneocyte components. The final massive signal is triggered by the stimulation of CaRs by the sharply increased external $Ca^{2+}$ level in the upper granular and transitional granular/horny layers. It drives the assembly of the accumulated components by a poised apoptosis mechanism to make a dead, keratin-packed, horny-shelled corneocyte.

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# 4

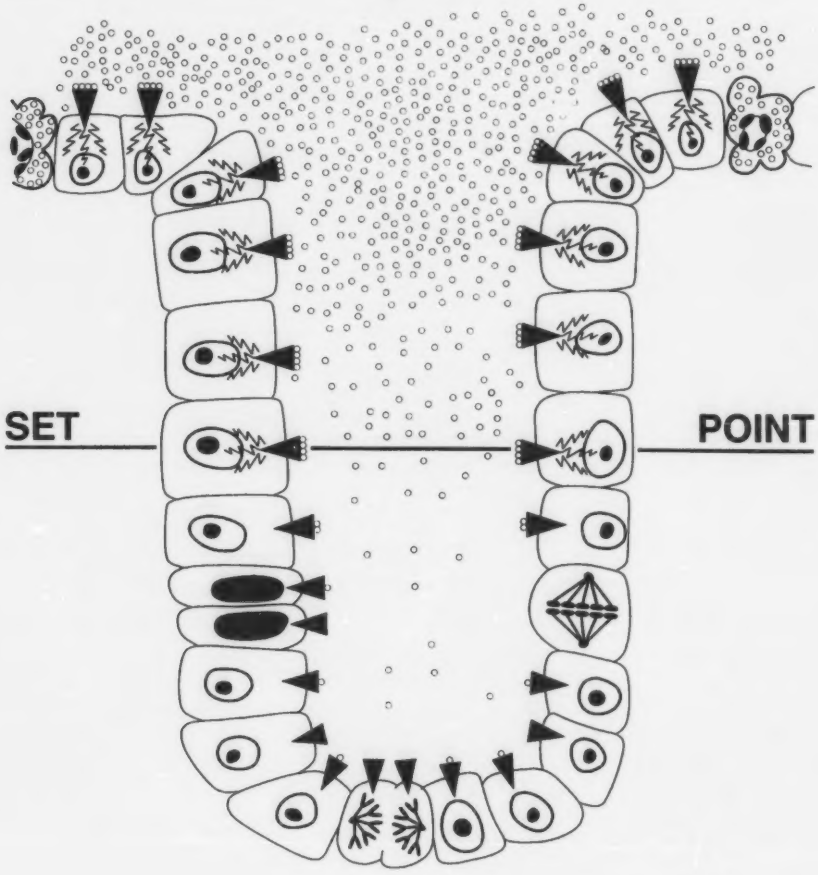
# Calcium and Colon Cell Diffpoptosis

## Colon crypts, stem cells, and a cellular escalator

According to the conventional model, the colonic counterparts of the skin's basal lamina-bound stem cells are a small number of clonogenic cells nestling at the foot of each monoclonally derived crypt shaped like the finger of a glove dipping down into the wall of the organ from the flat mucosal surface (Figs. 4.1 and 4.2).[1–5] A stem cell may divide symmetrically into two stem cells or two differentiation-bound, transit-amplifying daughter cells or it may divide asymmetrically into one stem cell and one differentiation-bound transit cell, but on the average, stem cells produce one stem cell and one differentiation-bound transit cell.[1,3] The first three generations of transit cells are also potentially clonogenic, but the later generations are proliferatively disabled and irreversibly committed to differentiate into three kinds of cell: absorptive cells, EC (enterochromaffin or enteroendocrine) cells, and mucus-producing goblet cells.[1–5] The EC cells are the colon's pressure sensors, which, when squeezed by fecal distension, release 5-HT (serotonin) to stimulate intrinsic sensory neurons with $5\text{-HT}_{1P}$ receptors that signal the motor neurons of the gut's massive "second brain" to start a peristaltic muscular pushing of the fecal mass forward.[6] The absorptive cells are the "work-horses" of the colon. They control the flow of water and electrolytes across the colon epithelium into the blood. They also are the cells with the transporters that limit the response to the sensor signals by taking up the 5-HT squeezed out of the EC cells.[6] (These absorptive cells can also cause a severe hypermotility and diarrhea when their 5-HT transporters are shut off by a SSRI (specific serotonin reuptake inhibitor) such as Prozak.)[6] The mucus cells provide the colon surface with a protective mucus coat and the $Ca^{2+}$-binding mucopolysaccharides for an intracryptal $Ca^{2+}$ gradient.

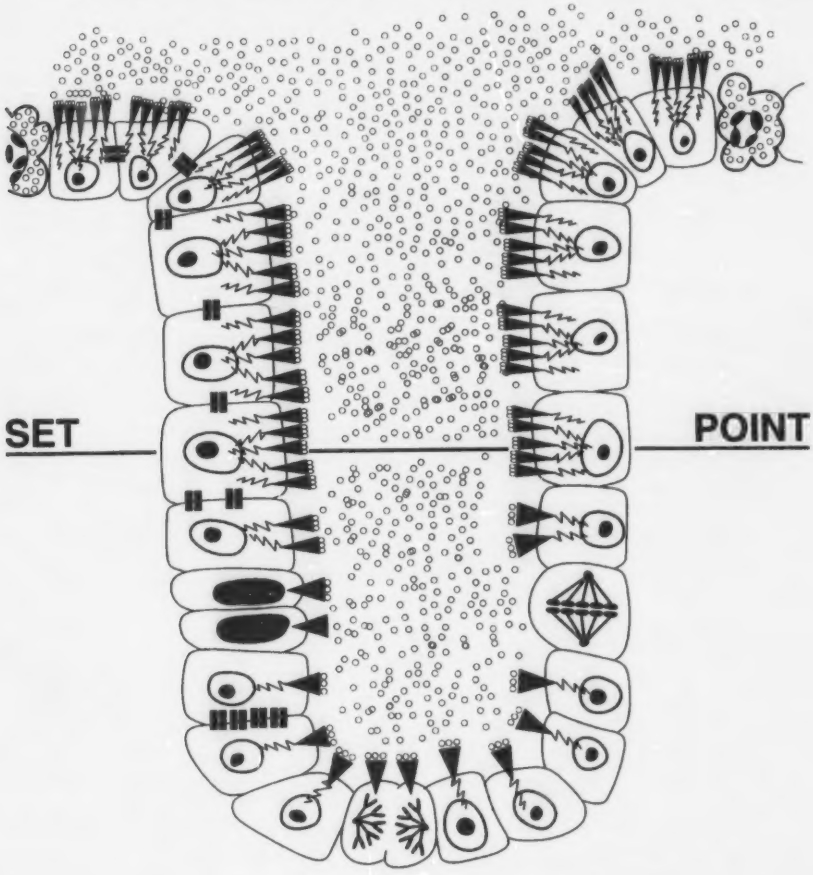
These mature, functioning, non-proliferating cells live for only a couple of days before dying and therefore must be replaced by the progeny of the small clone (one to four cells in the mouse) of pluripotent stem cells anchored to a specific niche at the bottom (cell positions 1 and 2) of each one of the millions of monoclonal, finger-like crypts.[3] The proliferative potential of the stem cell is probably maintained, and initiation of the diffpoptosis program suppressed, by signals from the same extracellular matrix-bound clusters of β1 integrins that maintain the proliferative potential and suppress diffpoptosis in its keratinocyte counterpart.[7,8] It is also maintained by signals from receptors for various autocrine, endocrine, juxtacrine, and paracrine factors from blood, neighbors, and nerve terminals. Like all cycling cells, these cells express the c-*myc* gene, the product of which can be either proliferogenic or apoptogenic, depending on the presence of survival signalers.[9–11] The expression of c-*myc* and thus its targets, the cycle-starting–driving

**Fig. 4.1.** One possible scenario for the control of colon cell proliferation and diffpoptosis by $Ca^{2+}$ and the CaR or CaR variant ($Ca^{2+}$, or more accurately, divalent cation receptors or sensors; solid triangles). In this version, there is an intracryptal $Ca^{2+}$ gradient (hollow circles) which triggers sensor signaling when the cell reaches a level in the crypt where the $Ca^{2+}$ concentration is at the receptors' set-point. The combination of fading proliferation-driving $\beta_1$ intergrin signals, surging CaR signals, emerging E-cadherin cell–cell tethers, and increasing APC expression stop cell cycle initiation, drive the upward gliding, differentiation and finally apoptosis of the absorptive cells on the flat mucosal surface.




cyclin D1 and CDK 4 genes, are ultimately due to the promotion by β-catenin·Tcf-4 of the formation of transactivator complexes on the c-Myc gene's enhancer region.[12,13] The importance of β-catenin·Tcf-4 for the maintenance crypt cell proliferation and thus the colon epithelium is demonstrated by the absence of proliferating cells in the colon crypts of *Tcf4*$^{-/-}$ mice that die at birth because of a badly depleted, stretched, and broken mucosal epithelium.[12] The proliferation of cells in the early stages of intestinal epithelial development is driven by other factors, but the β-catenin·Tcf-4 mechanism takes over as crypts appear and then remains a major player in the control of crypt cell proliferation.

**Fig. 4.2.** Another scenario for the control of cell proliferation and diffpoptosis by $Ca^{2+}$ and the $Ca^{2+}$ sensors in the colon crypt. In this scenario, there is no significant intracryptal $Ca^{2+}$ gradient, but at a certain point in their journey up the crypt wall the cells increase the sensor density at which their signaling starts contributing to the cells' differentiation and ultimate apoptosis.




Unlike their counterparts in the benign world of the small intestine, the colon crypt cells must protect themselves from premature death induced by exposure to swarms of fecal bacteria and countless noxious agents, including mutagens. They do this with the anti-apoptotic Bcl-2 protector protein, the expression of which is confined to the stem cell region in the base of the crypt.[3,14–16] While this is essential for keeping a healthy functioning mucosa in a hostile environment, it has the not inconsiderable *disadvantage* of also protecting neoplastic mutants from the apoptogenic weapons of the mutation-detecting gatekeepers such as p53(TP53). This is why there are colon cancers but virtually no small intestine cancers.

Stem cells can somehow detect the loss of one of their members as well as the loss of more distant cells.[3,17] This loss stimulates the stem cells to cycle faster and shift from asymmetric to symmetric divisions, and it stimulates the recruitment of "potential" stem (i.e., clonogenic) cells in the crypt back into the proliferative pool to replace the lost

cells.[1,3] How do they detect cell loss? Perhaps cell loss simply increases the available space (cellular Lebensraum) on the basal lamina for integrin-primed proliferation. However, it is likely that it is the intercellular canalicular or gap junctional communication network that provides the measure of the size of the colon cell population.[18] A loss of cells would change the volume of proliferative modulators flowing through the gap junctional network.[18,19] In murine small intestinal crypts, there is an efficient transfer of microinjected Lucifer Yellow dye between cells at the base of the crypt, but the transfer efficiency drops between the upper middle and the top of the crypt. There is a similar network in the colon,[20] which would account for the spreading of an internal $Ca^{2+}$ surge from cells at the base of a colon crypt to cells in the mid-region of the colon crypt when the $M_3$ muscarinic receptors of the basal cells are stimulated by acetylcholine.[20] The activated $M_3$ receptors stimulate the generation of $Ins(1,4,5)P_3$, which is probably what passes through the gap junctions from cell to cell, releasing $Ca^{2+}$ from internal stores as it goes. Indeed, a similar upwardly spreading $Ca^{2+}$ wave can be started by microinjecting $Ins(1,4,5)P_3$ into the basal crypt cells.[20] Clearly, a loss of cells would reduce the volume of intercellular traffic of proliferation moderators and increase the accumulation of proliferative promoters in the network's remaining cells.

Interestingly, just such a cryptal gap junctional network has been assigned the central role in a radically new model of the control of intestinal cell proliferation and differentiation.[21] According to this iconoclastic model, *all* of the columnar cells in a crypt have the same proliferative potential as the classical stem cell, and they will proliferate if the concentration of an intraepithelial growth factor that is small enough (1 kD or smaller) to pass through gap junctions exceeds a certain level. Hence, there are no distinctions between actual and potential stem cells, transit amplifying cells or mature cells as there are in the conventional model.[1,3,22] In other words, there would be no asymmetric division of stem cells. The key feature of this model is that the growth factor is produced *only* by "stem" cells in the lower part of the crypt from which it would pass up the chain of cells lining the crypt through gap junctions. At a certain level in the crypt, the concentration of the factor would drop below a threshold level because of dilution and decay. The cells at this level and higher would not be able to proliferate, and the differentiation program would start either by default or be triggered by signals from the CaRs to be discussed further on. Loss of cells from the chain or an increase in the production of the growth factor or the number of growth factor producers would cause a build up of the growth factor and an extension of the proliferative zone to higher levels of the crypt.

As we said above, according to the conventional model a true stem cell on the average (although not necessarily) divides asymmetrically at the end of its cycle into another pluripotent stem cell, which stays in the niche, and another daughter cell, a *potential* clonogenic stem cell, which can still proliferate indefinitely but not unless prodded to do so by something such as a severe loss of cells that requires the recruitment of more stem cells[1,3] or possibly the mutational disabling of a proliferation-monitoring/restraining gatekeeper gene. From the second or third generation onward, a cell cycle counter starts working in the rapidly cycling transit cells, and when the number on the dial reaches "0" they stop cycling. By this time, they have left the proliferative zone crafted by the combined actions of integrins, basal laminar components, and factors from their assigned pericryptal fibroblast escorts.[22] In the mouse, a transit amplifying cell of only limited clonogenic potential, and its descendants will complete eight cycles to produce a clone of

256 terminally differentiated cells. Like a transit amplifying basal keratinocyte, it expresses a set of β1 integrins, the signals from which prime its proliferative response mainly to signals from growth factors such as autocrine/paracrine TGF-α that start the cresting and crashing cell cycle-driving, stage-specific CDKs while moving away from the natal niche.[8,23] The proliferative activity may also be modulated by the circadian ebb and flow of other factors such as EGF and gastrin from the upper gastrointestinal tract and adrenergic, cholinergic, and other agents from the neuronal nets of the gut's "*second brain*" enveloping the crypt.[6,24–28] This precursor cell and its progeny, expressing such proliferation-related genes as cyclin D1, c-*myc*, and c-*myb* and protected from premature apoptosis by the Bcl-2 protein, glide along the basement membrane and up the crypt wall.[3,24–30] However, although the lower crypt is a place of intense cell production, it is also a minor apoptotic death zone, although there is much less spontaneous apoptosis here than in the small intestinal crypts.[3,31–32] While this cell death could be meant to prevent the propagation of cells with damaged or misrepaired DNA, its main purpose is simply to prevent cellular overproduction. Thus, for example, if a stem cell in an undamaged crypt should happen to divide symmetrically into two stem cells there could be a considerable cellular overproduction. This overproduction would shrink the amount of available space on the basal lamina, the cellular *Lebensraum*, which would force an actively cycling cell expressing the potentially apoptogenic c-*myc* to detach from the basement membrane and cut off its integrin-mediated and orchestrated "survival signals."[9–11] This would trigger c-Myc-mediated anoikis (detachment-induced apoptosis)[33] and the prompt disposal of the remnants of the dead cell by its neighbors.[7]

The epithelial cells are assigned a pericryptal fibroblast escort to accompany them up the crypt wall. Indeed, the location of the fibroblasts' proliferation, differentiation, and killing zones and their rate of migration up the crypt wall are the same as those of the epithelial cells.[34–38] There is undoubtedly a continuous exchange of information between the fibroblasts and epithelial cells in the form of factors and collaboratively made changes in basement membrane constituents.

## $Ca^{2+}$ and the control of colon cell proliferation, differentiation, and apoptosis

The cellular production in a colon crypt is so enormous that the crypt populations are turned over completely every 3 days in humans and mice.[1,1a,5,31] Each crypt in a mouse's proximal colon contains about 100 proliferating cells, which generate about 6 new cells per hour, while in the distal colon there are about 200 proliferating cells per crypt, which generate about 21 new cells every hour.[1,1a,5] Each of the millions of crypts in the human transverse colon produces 2.7–8.8 cells per hour.[31] Obviously, there has to be a culling mechanism to keep the cell population at a constant size to avoid overgrowth. Until recently, it has been assumed that the population is kept in check by the shedding of cells from the flat surface epithelium of the colon or at the tips of the villi in the small intestine. However, it now appears that colon cells are eliminated by apoptosis and the eating of their remnants by their neighbors at the bottom of the crypt; by apoptosis, cannibalism, and shedding at the top of the crypt; and in the small intestine, by apoptosis and cannibalism by neighbors at the bottom of the crypt, and by apoptosis and then by engulfment and (or) shedding at the villar tip. (The reason why this has not been realized

until now is simple — the swift disposal of apoptosing cells by their neighbors and macrophages.) Therefore, differentiation in another continuously renewing epithelium is really differentiation-apoptosis or diffpoptosis. But in this case, the apoptotic mechanism does not make the ultimate functioning cells as it does in the skin by converting component-preloaded keratinocytes into functional corneocytes. Instead, it eliminates the colon cells after they have matured and functionally peaked and thus prevents overpopulation with cellular seniors that have passed their peak.

Does $Ca^{2+}$ affect the proliferation of colon stem and transit-amplifying cells as it does the proliferation of basal keratinocytes? Sadly, there is very little information about $Ca^{2+}$ and colon cell proliferation even in the plastic world of the culture dish at least in part because *normal* colon cells respond badly to the disruption of their β1-integrin basement membrane linkages.[7] Breaking these linkages in order to isolate the cells for culturing triggers anoikis, which kills them within as little as 4 h unless they can rapidly reattach to a collagen substrate and restore signaling from integrin-growth factor receptor clusters.[7] Nevertheless, it seems that $Ca^{2+}$ affects colon cell proliferation in the same way it affects keratinocyte proliferation. Freshly isolated normal human colon cells need about 0.1 mM external $Ca^{2+}$ to proliferate optimally, while their proliferation and the proliferation of rat colon cells is stopped and the cells are induced to differentiate by the high (0.8–2.0 mM) $Ca^{2+}$ concentrations that can be found in the fecal water.[39–42] $Ca^{2+}$ also controls proliferation in the real world of the crypt where the cells need some $Ca^{2+}$ to proliferate maximally but cannot do so with smaller or larger amounts of the ion.[40] Thus, the proliferative activity of cells in crypts from normal rats given a diet containing either 0.5 or 15.0 g of calcium/kg was significantly lower than the optimal activity in the crypts from rats given a diet containing 5.0 g of calcium/kg.[43]

The key, and extremely important, question is whether there is a top-down $Ca^{2+}$ gradient in the colon crypt like the epidermal gradient (Fig. 4.1). The short answer is that we don't know yet. Unlike the air-exposed skin, where the $Ca^{2+}$ gradient has to be produced by keratinocytes bringing the ion up from the blood at the bottom and dumping it out at the top during their conversion to corneocytes, there is already a high $Ca^{2+}$ concentration in the fecal stream flowing by the mouth of the crypt, which enables direct top-loading. Obviously, from the evidence we do have, if the $Ca^{2+}$ concentration at the bottom of the crypt were to equilibrate with the millimolar concentration in the fecal stream by $Ca^{2+}$ diffusing down from the crypt mouth it would raise the external $Ca^{2+}$ well above the optimal level for the proliferation of the stem and transit amplifying cells. And loading the diet with a large amount of $Ca^{2+}$ does shrink the cryptal proliferative zone (i.e., decreases the value of Φ, the % of cycling cells in the upper 40% of the crypt).[44–49] As suggested by Whitfield,[25,26] a $Ca^{2+}$ gradient could be established by a bottom-to-top gradient of $Ca^{2+}$-binding chondroitin sulfates, heparin sulfates, and particularly the sialo- and sulfomucins secreted by the goblet cells that are concentrated in the lower regions of the crypt.[20,50–56] Indeed, the distribution of mucins (particularly sulfomucins) parallels the proliferative activity in the crypts of human and rat colons.[20,52,55] Lower crypt cells, like basal and spinous keratinocytes, may also help reduce the lumenal and intercellular $Ca^{2+}$ concentration by accumulating and sequestering the ion or dumping it into the blood, while upper crypt cells may oppose downward $Ca^{2+}$ diffusion by actively accumulating the ion. Brenner et al.[57] have recently reported a striking bottom-to-top *intracellular* $Ca^{2+}$ gradient in mouse colon crypts and a crypt level-dependent responsiveness to a change in

the extracellular $Ca^{2+}$ concentration and have mentioned that the same gradient has been found in human crypts. Thus, for example, the concentration of $Ca^{2+}$ in the cells of isolated whole murine crypts (determined by loading the cells with the $Ca^{2+}$-specific fluoroprobe FURA-2) rose from 70 to 83 nM in the lower crypt to 157 nM at the crypt mouth when the crypts were in $Ca^{2+}$-free buffer. When the crypts were suspended in buffer containing 1 mM $Ca^{2+}$, the intracellular $Ca^{2+}$ rose from 262 to 297 in the lower crypt to 463 nM at the crypt mouth. The maintenance of this gradient required $1\alpha,25(OH)_2$ vitamin $D_3$ because it was lost in animals fed a low-vitamin $D_3$ diet, which indicates the involvement of the transcellular transport of $Ca^{2+}$ by the $1\alpha,25(OH)_2$ vitamin $D_3$-dependent protein calbindin from the lumen to the blood. It may also indicate the involvement of signals from the CaRs on the cells' apical surfaces,[58–62] because expression of parathyroid gland's CaR is promoted by the seco-steroid (at least it is in keratinocytes).[63]

According to Kállay et al.[63a] and Scheinin et al.[63b], only the chromogranin A-containing enterendocrine cells at the base of the crypts make this particular CaR in the human colon. This suggests that if the $Ca^{2+}$ concentration in the crypt should rise above a certain limit, the signals from the CaRs might cause the enteroendocrine cells to release a proliferative suppressor such as pancreastatin, a cleavage product of their chromogranin A.[63b] Alternatively or additionally, there might be a CaR-suppressible production of a mitogen, such as the diffusible one proposed by Gerike et al.[21], by enteroendocrine cells. However, the specific CaR may not be the only $Ca^{2+}$ sensor. The other crypt cells might have a different sensor (e.g., RyR, the ryanodine-sensitive receptor/channel)[58] because cultured colon cells without their enterendocrine companions are still proliferatively shut down by low $Ca^{2+}$ concentrations.[39–41,63c] Whatever they may be, these $Ca^{2+}$ sensors are most likely the agents through which external $Ca^{2+}$ controls the proliferation and diffpoptosis of the suprabasal colon cells (Figs. 4.1 and 4.2). Perhaps lower crypt cells in the normal man or mouse bearing a minimal number of $Ca^{2+}$ sensors and equipped with calbindin collect $Ca^{2+}$ with their calbindin and take it to their basolateral membranes and dump it into the passing bloodstream. This pumping mechanism plus the mucins could produce a low, proliferation-compatible level of external $Ca^{2+}$ just like the ECaBP-mediated uptake of the ion that we suggested in the previous chapter maintains the low, proliferation-compatible $Ca^{2+}$ level in the basal epidermis. At higher crypt levels, the cell's $Ca^{2+}$ sensor density might increase. The rising flow of PLC-activating, intracellular $Ca^{2+}$-moblizing signals from the receptors[61] would increase the steady-state intracellular $Ca^{2+}$ level. A variant of this model would be that the cell's $Ca^{2+}$ sensor density does not change during its journey up the crypt, but the cell's internal $Ca^{2+}$ surges when the luminal $Ca^{2+}$ level reaches a specific set point in the upper crypt. In either case, the more intense $Ca^{2+}$ sensor signaling and the rising internal $Ca^{2+}$ level would be enough to stop proliferation and start differentiation.

## Integrins, ILK, catenins, and proliferation

The proliferation of the precursor cells in the low-$Ca^{2+}$ base of the colon crypt, like that of the basal keratinocytes in the previous chapter, is driven by adhesion-induced clusters of $\beta1$ integrins and receptors for growth factors such as EGF and TGF-$\alpha$. The signals from these clusters stimulate PtdIns-3K, the PtdIns(3,4,5)$P_3$ product of which

stimulates intergrin-associated ILK and membrane-associated PKB.[64–68] ILK is connected to the β1 integrin cytoplasmic domain by the cysteine-rich LIM motifs of the PINCH adaptor protein, which also attach the Nck-2 adaptor protein that connects to various growth factor receptors through its SH2 and SH3 domains.[69,70] These signaling complexes keep the cell in the proliferative mode by increasing the activity of the E2F-family of proliferogenic transcription factors when PKB activates the Cdk4·cyclin D protein kinase that hyperphosphorylates and thus inactivates the E2F-suppressing Rb-family pocket proteins).[71] They also prevent the destruction of β-catenin, which can get into the nucleus to stimulate key proliferation-driving genes such as those for Fra-1 and c-Jun (components of AP-1 transcription complexes), but most importantly the genes for cyclin D1 and the master transcription factor for several replication genes, c-*myc*.[12,72–74] The c-Myc product heterodimerizes with Max protein to form Myc·Max complexes, which, among other things, stimulate the expression of the gene for the Cdc25A protein phosphatase that also activates the $G_1$ buildup, starting Cdk4·cyclin D1 protein kinase.[9] The complexes also stimulate the expression of TERT, the telomerase catalytic subunit, which if c-Myc expression should persist and TERT should exceed a certain level would contribute to cellular immortalization and tumorigenesis.[75] Premature apoptosis is prevented by the cells making sure that c-Myc, the E2F1 expression it stimulates, the apoptosis-triggering p53(TP53) that excessive stimulation of E2F1 can in turn stimulate, and Cdc25A are kept within the proliferogenic realm using survival signals from autocrine IGF-I, which stimulate the expression of the anti-apoptosis Bcl-2 protector protein, and by suppressing the expression of pro-apoptosis proteins such as BAD.[9,67,72,76,77] ILK and PKB also disable GSK-3β,[67] which prevents the protein kinase from marking β-catenin for ubiquitination and consignment to the proteasome shredder and downregulating the cycle-driving cyclin D1.[73,78–81] The signals from the β1-integrin-assembled complexes might also suppress the expression of the APC (adenomatous polyposis coli) protein that joins with another protein, axin or conductin (axil), to bring β-catenin into contact with GSK-3β and ultimately the shredder.[81,82] They may also suppress the expression and display of the $Ca^{2+}$-dependent E-cadherins, which reduce the availability of β-catenin for gene stimulation by using it to link the E-cadherin C-terminal tails to α-catenin, vinculin, α-actinin, and F-actin to make the intracellular parts of the cables with which the $Ca^{2+}$- binding cadherins tie neighboring cells together.[83–85]

The proliferation of the colon stem and transit amplifying cells also requires cAMP signals, a predominance of the proliferation-promoting PKAI (type I cAMP-activated protein kinase) over the differentiation-promoting PKAII,[86,87] and expression of the 320–350 kDa Ki-67 protein, which is a universal marker for all proliferating cells, including colon cells.[48,88–90] The proliferation-driving signals from the integrin·growth factor receptor complexes increase the expression of the RI (type I regulatory) subunit gene and reduce the expression of the RII subunit gene.[86,87] We shall soon see that a reversal of the expressions of the RI and RII genes is part of the colon cell's diffpoptosis program.

## $Ca^{2+}$, E-cadherins, APC, and diffpoptosis

As an epidermal basal cell moves away from the natal cluster, its β1 integrin complement drops and its attachment to the basal lamina weakens. Eventually, the integrin

signaling drops below a critical level, which triggers a $Ca^{2+}$ signal that triggers diffpoptosis. We know that colon cells reduce the expression of their β1 integrins as they move toward the top of the crypt and the colon surface,[7,8] but without a reliable in vitro model system we do not know whether there is a $Ca^{2+}$ surge associated with the integrin shift like that which occurs in keratinocytes. The fading signaling from β1 integrins and their associated growth factor receptors, and the PtdIns-3K/ILK/PKB activities they stimulate, derepresses E-cadherin gene expression and increases GSK-3β activity, which, respectively, sequester and destroy β-catenin and thereby shut down the expression of the catenin's cycle-driving target genes. However, colon cells do not detach from the proliferative zone and lift into the differentiation and killing zones, as do keratinocytes. Instead, colonocytes stay attached and glide along the basement membrane in a chain (a cellular escalator) as they go from the proliferative zone in the lower third of the crypt to the differentiation zone and finally the killing zone. The cell can no longer make cycle-driving c-Myc·Max transcription complexes, which start the events leading ultimately to the waves of cyclins E and A for the Cdk2·cyclin protein kinases needed to make the replication factories. But as if this were not enough to stop cycling, the upwardly moving colon cell, like a detaching keratinocyte, also abruptly stops expressing the Ki67 protein, reduces the expression of the RI subunits of PKAI, increases the expression of the RII subunits for the differentiation-promoting PKAII,[86,87] and possibly because of an increasing volume of signals from the β4 subunits of emerging differentiation-specific integrins (such as α6β4),[91] starts expressing the $p21^{Cip1/WAF1}$ CDK inhibitor, which prevents the $G_1$ buildup of materials for the replication factories by inhibiting Cdk4·cyclin D and Cdk2·cyclin E protein kinases as well as any existing Cdk2·cyclin A protein kinase.[88–97] This p53(TP53)-independent (like that in keratinocytes) burst of $p21^{Cip1/WAF1}$ expression may be used to shut down the cell cycle without triggering apoptosis, which would allow the cell to differentiate and function until it reaches the killing ground.[95–97] p21's job is to stop the colon cell cycling without being involved in starting differentiation.[97] After it has done its job of silencing the Cdk·cyclins, it drops down to a lower level,[97] which is still high enough to prevent any resurgence of the Cdk·cyclins without interfering with differentiation as its does in keratinocytes.[98] The surge of p21 is followed by a surge of another multifunctional CKI, $p27^{Kip1}$, which, despite it being a CDK inhibitor, is probably involved in differentiation rather than cell cycle suppression[97] as it is in keratinocytes.[98]

The developing $Ca^{2+}$-dependent, E-cadherin-mediated cell–cell adhesion might be responsible for the enhanced expression of TGF-β1 and TGF-β2 and the type II component of the TGF-βs' Type I·Type II receptor complex in the upper part of the crypt.[99–101] The autocrine/paracrine TGF-βs are responsible for the producer cells expressing the $p15^{INK\,4b}$, $p21^{Cip1/WAF1}$, and $p27^{Kip1}$ CKIs and suppressing the expression and activities of CDKs and shutting down c-*myc* in the upper, differentiation zone of the crypt.[96–102] In other words, the TGF-βs may throw the switch that turns off proliferation when the cells reach the differentiation zone. They also stimulate their producer cells to make differentiation-specific matrix-binding integrins, and these factors diffuse over to the escorting fibroblasts and cause them to make the matrix components that bind and activate these integrin receptors.[103–105]

The development of a proliferatively disabled functioning polarized columnar epithelial cell with the expression and placing of enzymes, channels, and other components

at appropriate places in the apical micovillar and basolateral membranes requires the continued actions of different integrins plus several emerging cell–cell and cell–matrix adhesion components, starting with E-cadherins. As in the keratinocytes of the previous chapter, extracellular $Ca^{2+}$ puts the emerging E-cadherins into their active configuration. Their cytoplasmic domains combine with β- (or γ-) and α-catenins to form complexes that link them to α-actin, ankyrin, and other components of the cytoskeleton as well as to enzymes such as $Na^+/K^+$-ATPase.[84,106,107] These complexes include receptor kinases such as the EGF/TGF-α receptor that binds to the conserved core region of β-catenin[108] and from this vantage point can drive various functions, including the expression of differentiation-specific genes. Using the establishment of functional polarity in Madin–Darby canine kidney cells as a model, it is likely that the formation of tight junctions and desmosomes by the differentiating colon cells is started and driven by global and localized $Ca^{2+}$ surges triggered by the cell–cell contact and the $Ca^{2+}$ sensors.[109–112] These surges drive the translocation of junctional components to appropriate assembly sites on the cell membrane to form tight junctions and desmosomes. The result is tight, $Ca^{2+}$-driven, cell–cell adhesion by cytoskeleton-linked signaling complexes, which reconfigures and polarizes the now-proliferatively silenced cell and puts clusters of key enzymes at particular regions of the cell surface such as disaccharidases, peptidases, $Na^+$-dependent glucose co-transporters in the apical brush border and $\alpha_2$- and β-adrenergic receptors, VIP (vasoactive intestinal peptide) receptors, $Na^+/K^+$-ATPase, and outwardly directed $Na^+$-independent glucose transporters on the basolateral border.[109–112] Other stem cell progeny become goblet cells, most of which stay near home in the lower crypt to make mucins for the $Ca^{2+}$-binding shield.

A major player in colon cell maturation is the APC (adenomatous polyposis coli) tumor suppressor protein, the gene for which is mutationally disabled or eliminated during the progression of a colon cell's progeny to carcinoma. As colon cells mature, they express and accumulate the 312-kDa APC phosphoprotein.[113] APC seems to have at least three roles in diffpoptosis, two of which involve association with the cytoskeleton and cell–cell adhesion.[114–120] First, as we have learned, APC associates with, and is phosphorylated by, GSK-3β on an axin (or conductin) platform, which enables it to bind β-catenins in the lateral cytoplasm and compete with, and thus directly modulate, cell-tethering by the E-cadherins.[66,81–83,106,107,117–119] APC also strongly promotes microtubule assembly with its C-terminal region and collects in clusters near the ends of cytoplasmic microtubules.[115,116] There it promotes the formation of junctional complexes and signaling as well as various microtubule-mediated functions, which include moving the cell up the crypt wall toward the killing zone. Obviously, APC's cell-moving and $Ca^{2+}$·E-cadherin's cell-tethering actions must be balanced to produce the moving chain of tethered functional cells. Indeed, upsetting this balance by hyperexpressing E-cadherin would, and does, jam the "escalator." Finally, the APC-bound "excess" β-catenin on the axin platform is marked for ubiquitination by the GSK-3β kinase and sent to the proteasome shredder.[81] In this way, APC keeps β-catenin at a low level.[121–125] This is needed to balance cell production and diffpoptosis because any free β-catenin would enter the nucleus where it would displace the Groucho and CtBP transcription co-repressors and bind to enhancer-bound Tcf-4 factors to form active transcription complexes on the enhancers of cell cycle-driving genes, which would extend the proliferative zone and oppose differentiation and apoptosis.[12,72,76,121–125]

Another key player in colon cell maturation is DCC (Deleted in Colon Cancer), which, as its name indicates, is lost during the later stages of the progression to malignant colon cancer. DCC has been considered as a cell–cell adhesion protein belonging to the N-CAM (Neural Cell Adhesion Molecule) family.[126–128] However, from the outset there was something wrong with this idea because there is not enough of it to be a cell adhesion factor. We now know that it is a *receptor*, which, when activated by contacting the laminin-like netrin-1 in the extracellular migration trackways with its Ig-like and fibronectin-type III extracellular motifs, sends signals (that involve G-proteins and $Ca^{2+}$) into the colon cell through its cytoplasmic domain, which suppress anoikis and somehow promote differentiation (possibly by inhibiting CDK activity) and guide the differentiating cell's migration up the crypt wall just as neurons use their DCC receptors to steer growing neurites toward their targets.[126–130] However, DCC is also part of an important "fail-safe" mechanism that guards against a cell being able to detach from the basal lamina with impunity and metastasize to distant places.[131] Thus, the apoptogenic mechanism is silent when the cell is attached to the basal lamina and DCC is attached to its ligand and presumably steering the cell along the trackway and emitting an anoikis-suppressing signal. Should the colon cell try to detach itself from the trackway, there would be an increase in caspase-3 activity, which, by cleaving it at $Asp^{1290}$, converts DCC into a potent apoptosis driver.[131]

Once again, a $Ca^{2+}$ surge has been cited as the common mediator of the action of another differentiation factor. However, we must take the opportunity at this point to remind the reader that in our current primitive state of knowledge that all $Ca^{2+}$ surges are not the same. These several regulators and adhesion complexes trigger different space–time–frequency-modulated $Ca^{2+}$ transients of various shapes and sizes in different parts of the cell. These specifically patterned streams of transients are the words and phrases of the complex $Ca^{2+}$ language that different agents use to tell cells how to reconfigure themselves into mature absorptive or goblet cells.[132]

As the DCC-directed, upwardly gliding cell is proliferatively shut down by the appearance of TGF-βs and their receptor complexes, the fading of proliferation-related integrins and the cadherin-triggered/mediated adhesion to its neighbors, it of course shuts off its c-*myc* and c-*myb* genes.[27,28,133] It might be expected that as it moves up the crypt and stops cycling, the differentiating colon cell would also stop making its principal autocrine proliferogen, TGF-α. But it doesn't! It keeps making TGF-α and the EGF/TGF-α receptors.[134] However, these receptors are probably now activated at least in part by the EGF-related amphiregulin, which is expressed only by differentiated columnar epithelial and goblet cells.[135] Because the receptor signals may be different and the signals' old targets have been replaced in the functionally reconfigured cell by new diffpoptosis-related ones, neither the emerging amphiregulin nor the TGF-α made at the top of the crypt can make the maturing cell start cycling.[134]

In the skin, basal cells express the PTHrP gene at a low level and display PTHrP receptors, but they do not translate any PTHrP messages into PTHrP holoprotein or produce their characteristic selection of PTHrP fragments. However, when a cell enters the spinous layer, PTHrP gene expression surges and the cell now translates the messages into PTHrP proteins, only to stop again when it enters the granular layer. Rat colon cells also express their PTHrP receptor gene(s) and probably make receptors and produce PTHrP mRNA at all crypt levels, but they translate these messages into PTHrP proteins

only at the top of the crypt and on the flat epithelial surface.[136] Similarly, PTHrP mRNA is made all along the crypt and villus in rat jejunum, but the messages are translated into PTHrP proteins only when the cells leave the crypt and start moving up the villus.[136] The functions of PTHrP and its fragments in the cells of the colon's surface epithelium and the small intestinal villi are unknown, except that PTH/PTHrP receptor signals can stimulate $Ca^{2+}$ transport by chicken small intestine cells.[137] However, while the keratinocyte's particular PTH/PTHrP receptors are coupled to phospholipase-Cβ, but not to adenylyl cyclase, the colon receptors are more conventionally coupled to adenylyl cyclase[138] and almost certainly to phospholipase-Cβ. PTHrP and its fragments are undoubtedly very important autocrine/paracrine factors in the mature colon epithelium just as it is in many other producer tissues. Significantly, the translation of its mRNA coincides with the entry of the cell into the apoptotic or killing zone[31,32] and the activity of the $Ca^{2+}$ sensors as they meet the very high $Ca^{2+}$ levels at the top of the crypt. It seems likely that PTHrP's job is to hold off terminal apoptosis as it does in cartilage by stimulating the expression of the Bcl-2 protector protein.[139]

When the colon cell nears the mouth of the crypt and the edge of the $Ca^{2+}$-rich fecal stream, it, like the granular keratinocyte, almost certainly hits a wall of external $Ca^{2+}$ and is battered by signals from its apical $Ca^{2+}$ sensors (Fig. 4.1). This signaling would expel $Ca^{2+}$ from internal stores, which in turn would open SOCs (stores-operated channels) and start an inward flow of external $Ca^{2+}$.[61,140] This could be why upper crypt cells (like the upper granular keratinocytes in the previous chapter) have the largest amount of intracellular $Ca^{2+}$.[57] These signals would complete the maturation of the cell. As in the granular layer of the epidermis, the rising intensity of signaling from $Ca^{2+}$ sensors could also be responsible for the large drop in gap junctional intercellular communication in the upper crypt.[20,21] But these signals also prepare the cell for something else — apoptosis.

Now we must revisit the APC tumor-suppressor protein, which will be at the center of the stage in the next section. So far, this protein and E-cadherin have been keeping the free β-catenin pool at a low level to promote cell maturation and the chain of mutually tethered cells gliding along the basal lamina without re-starting the proliferogenic genes that would disrupt the chain. When APC builds up beyond a certain critical level as the cell nears the top of the crypt,[113] it could weaken the E-cadherin-mediated linkage of the cell to its neighbors by dropping the β-catenin content below the level needed to make and maintain the tethering cables. The cell would soon break away from the chain and start the population-limiting apoptogenic mechanism.[31] Indeed, Morin et al.[141] have found that introducing a normal, active APC gene in colorectal carcinoma cells with disabled APC genes kills the cells by apoptosis. At this advanced stage of its life history, the cell starts getting ready to kill itself. It stops making the anti-apoptogenic Bcl-2 protein that protected its ancestors from premature death during their youth when they were at the bottom of the crypt and suicidally starts making the pro-apoptosis BAD, Bax, and Bcl-$X_S$ proteins.[3,13–15]

Now the end is near and the aging cell starts accumulating DNase I[142] and transglutaminase, which will eventually be used to convert it into a hardened corneocyte-like squame that is either swiftly engulfed by its neighbors or falls off into the passing fecal stream.[143,144] However, we must not forget the fibroblasts that have faithfully escorted the epithelial cells and helped nourish and drive their growth and differentiation during the journey up the crypt. They too, like the wives and retainers of an ancient King, must

apoptose and be swiftly eliminated by macrophages to balance the intense cell production going on in the lower crypt.[31]

## Colon cancer

### *The beginning of the road to cancer — aberrant crypts, polyps, and a loss of $Ca^{2+}$ control*

The steady battering of the colon with carcinogens over the years results in as many as 50% of western people harboring adenomas in their colons by 70 years of age. These carcinogens produce a genomic variability that a cell and its progeny can draw on to evolve by classical Darwinian natural selection strategies that enable them to survive and flourish despite damaged genomes and the best efforts of checkpoint guardians, cycle suppressors, and apoptogens.[145] Colon carcinogenesis, like carcinogenesis in other tissues, starts with genomic damage. In the proximal colon, bile acids are principal carcinogens. The N-nitrosylated conjugated bile acids are analogs of methylating carcinogens such as MNNG (*N'*-methyl-*N'*-nitrosoguanine) and MNU (*N*-nitrosourea), which start the carcinogenic ball rolling by methylating cytosines, which when deaminated to thymine causes mismatching G/C→G/Me-C→G/T mutations that triggers mismatch repair.[145,146] In the distal colon, BAFs (bulk adduct formers) are the principal carcinogens, which cause chromosomal rearrangements, loss or translocation of parts of chromosomes or loss of entire chromosomes, i.e., chromosomal instability and aneuploidy.[145] The cell tries to remove the adducts by cutting them out (nucleotide excision repair), but these molecular scissors can make things worse by too much chromosome cutting! Since Darwinian success is measured only by the enhanced ability of an individual cell to survive and replicate its genome, the intense selection pressure in a transforming population of cells will favor those clones that can override the growth arresting/repair mechanisms and proliferate despite their damaged chromosomes and aneuploidy. The pressure will then be on inactivating or getting rid of the cycle-suppressing pocket proteins and Cdk4/6·cyclin D-suppressing CKIs by disabling their genes by mutation or shutting them down by methylating the cytosines in the CpG/GpC islands in their 5'-regulatory regions, and hyperexpressing key cycle drivers.[145] And last, but not least, will be the disabling or elimination of the apoptogenic p53 (TP53) and mitotic checkpoint mechanisms. The result of this successful evolution by natural selection is a colon tumor and ultimately carcinoma in the cells of which about 500 of the 300 000 gene transcripts from about 45 000 different genes are expressed at significantly different levels from those in normal colon cells.[146]

However, while the world's focus has been on gene mutations as the agents of carcinogenic progression, there is something else which is probably equally important. This is the methylation of the cytosines in CpG islands in the promoters of the genes for growth suppressors and differentiation promoters.[145,147] As we learned in Chap. 1, cytosine methylation by DNA-MTase (DNA methytransferase) is a kind of molecular "not-to-be-read" Post-it® note that is put on to genes just after replication to duplicate their mother cell's functional characteristics. Since the genes for growth (tumor) suppressors and differentiation promoters have CpG islands in their promoters, they can be as permanently

and as replicably silenced by hypermethylation as by a disabling mutation. Thus, as we shall soon see colon cancers are the products of both gene mutation and epimutation (methylation).[146]

As a person ages, the DNA-Mtase activity in various tissues including the colon rises.[146] One of the effects of this in the colon is the hypermethylation of the CpG islands, and consequent silencing, of the estrogen receptor (ER).[146] An initiating mutation in cells with their silenced, epimutated ERs triggers their clonal expansion into the preneoplastic lesions to be described below.

The initiating genomic change caused by a N-nitrosylated conjugated bile acid in the proximal colon or a BAF carcinogen in the distal colon drifting down into the crypt from the fecal stream must happen in one of the actual stem cells or in a cell of the first two or three generations of still uncommitted, still clonogenically competent transit cells, but not in later rapidly cycling transit amplifying cells with their cycle counters running down, which eventually apoptose although they complete more cycles before doing so. Cancer is common in the colon, but rare in the small intestine.[3] Why? Mutagen-damaged cells in both colon and small intestine may respond to this chromosomal damage with p53(TP53)-triggered apoptosis.[95] It happens that the most apoptosis-prone cells in the mouse small intestine crypts are the clonogenic stem cells in position 4 where they do not contain the anti-apoptogenic Bcl-2 protein, but in the colon the most apoptosis-prone cells are only the non-clonogenic transit amplifying cells in the mid-crypt region (cell positions 11 to 12) far from the well-protected, Bcl-2-loaded clonogenic stem cells in positions 1 and 2.[3,16,17,148] The importance of Bcl-2 is demonstrated by the fact that in Bcl-2-null mice the level of spontaneous apoptosis rises significantly in colon, while there is no increased apoptosis in small intestine crypts.[3,16] Therefore, in the small intestine, mutagen-damaged clonogenic cells, unprotected by Bcl-2, kill themselves, while mutagen-damaged clonogenic colon cells protected by Bcl-2 survive as dangerous, self-renewing cells in which the clonal selection process has been started and are primed to respond to tumor promoters by proliferating rather than differentiating.

According to conventional wisdom, the mutated and epimutated human colorectal cancer clones appear in hyperplastic adenomatous polyps.[4,149,150] However, a large fraction of early lesions as well as advanced colorectal cancers have arisen from flat mucosa rather than polyps. In fact, the smallest detectable precursor lesions are clusters or foci of hyperplastic, distended, flask-shaped, branching, bifurcated (dividing) aberrant crypts.[150–177] These ACFs (Aberrant Crypt Foci) are the products an abnormal crypt cycle and stem cell expansion. An ACF starts with excessive clonogenic cell proliferation at the base of a crypt, which causes a branching or fission that moves up until there are two crypts, which in turn branch and divide to produce a crypt cluster.[2,155,158]

Some hyperplastic ACFs turn into, or perhaps start out as, the dangerous dysplastic adenomatous ACFs. The post-hyperplasia-generating mutations that start the carcinogenic process disrupt the differentiation program and extend the proliferative zone. In normal human colon crypts, the nuclear, proliferation-marking Ki67 and the hMSH2 mismatch-repair proteins are intensely expressed in the lower third of the crypt, but they are sharply downregulated and the $p21^{Cip1/WAF1}$ CKI upregulated when the tethered cells glide out of the proliferative zone. Even in the largest of the hyperplastic ACFs, proliferatively active cells expressing Ki67 and hMSH2 proteins were still restricted to the lower third of the crypts, while p21 was expressed only in the upper part of the crypts

and the surface epithelium.[96] However, in the carcinoma-hatching dysplastic ACFs, the mechanism(s) that shuts off Ki67 and hMSH2 expression and turns on the cycle-stopping $p21^{Cip1/WAF1}$ at the border of the diffpoptosis zone has been disabled, and the strict compartmentalization of cycling cells as indicated by the expression of Ki67 and hMSH2 is lost. Ki67 and hMSH2 are now expressed all the way up to the top of the crypt and the surface epithelium and overlapped with the expression of $p21^{Cip1/WAF1}$ in the upper regions.[96]

This extension of the proliferative zone into the differentiation and killing zones is the result of the initiated cells persistently producing autocrine/paracrine growth factors such as PDGF-B (platelet-derived growth factor-B), IGF-I, IGF-I variants, TGF-α, partly processed TGF-α precursors, and the EGF-like Cripto, which is not expressed by normal colon cells.[25,135,178,179] Because they can stimulate themselves with their hyperexpressed growth factors, the proliferation of the initiated/activated cells is not limited by the need for signals from adhesion-assembled complexes of β1 integrins and expogenous growth factors and their receptors. An example of the importance of a TGF-α-autocrine/paracrine loop for the proliferation of at least some colon carcinoma cells is the ability of a 23-base antisense oligonucleotide that recognizes TGF-α mRNA to reduce TGF-α secretion and proliferation of LIM1215 colon carcinoma cells.[180] Later, mutant cells appear which have either lost the Type II TGF-β receptor and are no longer susceptible to proliferative inhibition by TGF-β, or may be stimulated by TGF-βs or a paracrine factor such as PDGF-B induced by TGF-β.[99a,181–186] The mutants increase PKC-βII expression and reduce PKCs α and βI expression.[186a] They are also likely to start constitutively making PTHrP, which is known to stimulate adenylyl cyclase and proliferation of at least some initiated cells such as human LoVo colon carcinoma cells.[138] Indeed, an increased production of PTHrP is one of the earliest changes on the road to cancer and peaks in adenocarcinoma cells.[187] The possibility of PTHrP being an autocrine/paracrine enhancer of tumor progression that operates through the PTH/PTHrP receptor is suggested by an association of hyperparathyroidism with colon tumors.[188–191]

Excessive growth of normal, uninitiated cells is limited by anoikis[33] as well as cryptal branching and fission. An excess of cells would reduce the available basal lamina space, which would reduce or stop the flow anti-apoptosis signals from adhesion-induced β1-clusters. This would trigger anoikis and the swift disposal of the cadavers by their neighbors.[7,9] However, the carcinogen-initiated cells don't need signals from β1 integrins because of the persistent production of autocrine, PtdIns-3K-activating growth factors such as Cripto, TGF-α, IGF-I, and the resulting hyperexpression of cell cycle drivers such as the c-*myc* gene and inhibitors of apoptosis.[9,67,133,192] Another likely contributor to initiated cells' resistance to anoikis is the surging PTHrP, which may shield the cells by increasing the expression of the Bcl-2 protector protein.[139]

At the heart of colon carcinogenicity are the failures to effectively assemble the proliferation-suppressing, cell–cell/cell–matrix adhesion systems and express the TGF-βs and TGF-β receptors that switch on CKI genes and silence cell cycle genes such as those encoding cyclin D1 and c-Myc and to produce functioning columnar cells that ultimately apoptose. A common first step (in about 70% of all non-hereditary, non-germ line colorectal carcinomas) is a mutation in the first half of the coding region of the $Apc^{+}$ gene on the long (q) arm of human chromosome 5.[101,123] This starts neoplastic development.[149–193] It is a disabling of the *Apc* gene that is responsible for the hereditary

adenomatous polyposis coli from which the gene and its product got their name.[149–193] In this disease, the colon surface becomes studded with polyps, some of which spawn carcinomas.[149–195] In hereditary non-polyposis colon carcinoma (HNPCC) and some sporadic colon carcinomas, the *Apc* mutation is one of the many resulting from the disabling of the hMSH2 gene (on chromosome 2p 15-16) of the proofreading/editing mismatch repair system that scans newly replicated DNA for any single base-pair mismatches and multiple-base insertion–deletion loop-type misalignments, which it cuts out and replaces with correctly matched DNA.[22,196,197] However, in the rest of the non-germ line sporadic colorectal cancers the *Apc* gene is disabled even with a functioning mismatch repair mechanism.

Some ACFs, the relatively innocuous non-dysplastic ACFs, owe their hyperplasia to a mutant, constitutively hyperactive Ki-Ras protein, but they still have normal $Apc^{+}$ genes and are not neoplastic.[155,170–172,174,193] On the other hand, clonogenic cells in the dysplastic crypt foci are either homozygous $Apc^{-/-}$ or they have one normal $Apc^{+}$ and one disabled, $Apc^{-}$, gene. At least one APC mutant, specifically $Apc^{1309}$ in the central part of the gene, is a dominant negative, the product of which blocks the activity of the normal wild-type protein and is associated with a severe phenotype of an early appearance of thousands of polyps.[194] But despite this, $Apc^{-/-}$ crypts replicate faster into ACFs than $Apc^{\pm}$ crypts.[156]

Obviously, it is the loss of APC function that puts most clonogenic colon cells on the road to cancer? Why? How? $Apc^{+}$ is what Kinzler and Vogelstein[195] have called a *gatekeeper gene*, the product of which (like the basal keratinocyte's *Ptch* gene discussed in the previous chapter) prevents runaway proliferation. The answer lies in APC's ability to form a complex with axin (or conductin), the GSK-3β protein kinase, and β-catenin.[12,66,73,81,82,121–124,198] β-Catenin in this complex is phosphorylated by GSK-3β and thus marked for ubiquitination and proteasomal shredding, which stops it from getting into the nucleus to combine with the Tcf-4 architectural transcription factor and stimulate cell cycle drivers such as the c-*myc* gene.[12,64–86,121–124,198] However, if APC is disabled or lost, there will be enough β-catenin to promote proliferation and delay or prevent differentiation and apoptosis.[72–74,76,121–124,199,200] And diacylglycerol- / bile acid- / growth factor-stimulated upregulated PKC-βII in the initiated cells promotes the accumulation of β-catenin by phosphorylating and inhibiting GSK-3β.[186a] Indeed, while overexpressing β-catenin stimulates the expression of cycle-driving genes, it reduces the expression of a principal differentiation-related gene, the gene for ZO-1, the zonula occludens of the apical tight junctions that are among the key components of polarized functioning colon cells, and it would also result in a c-Myc-mediated hyperexpression of TERT, the catalytic subunit of telomerase, which could contribute to tumorigenesis by downregulating $p19^{ARF}$ and p53(TP53) and immortalizing the mutant cells.[75,186a,201] Thus, the cause of hyperproliferation and the expansion of the proliferative zone is too much β-catenin, only a fraction of which, at least in this early stage, can still be locked into cell-tethering cables by $Ca^{2+}$-E-cadherin. Indeed, some colon cancer cells still express a perfectly normal, functional APC protein and GSK-3β kinase, but they make a mutant β-catenin which cannot be phosphorylated and thus marked for the proteasome shredder by GSK-3β.[12,73,122]

However, there is a negative feedback mechanism that can mitigate the consequences of APC loss and excess β-catenin. β-Catenin·Tcf-4 also stimulates the

expression of Tcf-1, a close relative of Tcf-4 that shares gene targets with Tcf-4, but which cannot bind β-catenin. Because of this a surge of Tcf-1 can negatively feed back onto β-catenin·Tcf-4, compete with β-catenin·Tcf-4 for target genes, and limit the complex's ability to stimulate proliferation.[125] It follows from this that a loss of Tcf-1 during carcinogenesis could enhance the hyperproliferative consequence of APC loss.[125]

Although hyperexpression of β-catenin and its accumulation in the nucleus was first found to be responsible for starting colon carcinomas, it is now being found in other tumors. Thus, mutant non-phosphorylatable β-catenins are particularly prevalent in anaplastic thyroid carcinoma; and a large nuclear accumulation of β-catenin has now been found in the proliferating cells of 43% of human hepatocellular carcinomas, but not in the cells of adjacent non-tumorous (normal, cirrhotic nodular, dysplastic) liver tissue.[81,202] Deregulated β-catenin signaling has also been found in endometrial cancers, melanomas, ovarian cancers, pilomatricomas, and prostate cancers.[73]

One thing seems to be missing that could greatly help β-catenin drive proliferation and impede differentiation. As we learned in Chap. 1, the key to starting a cycle rather than shifting out of the proliferative mode is the expression of Cdk4·cyclin D, which hyperphosphorylates and inactivates the Rb-family pocket proteins that suppress the expression of replication-driving genes. Indeed, *most* cancers of continuously self-renewing epithelia have disabled their Cdk4·cyclin D1-inhibiting $p16^{INK\ 4a}$ protein, a consequence of which would be increased inactivation of Rb-family proteins.[202a,203] Another result of the inactivation or deletion of the INK 4a locus would be a loss of the locus's alternative product $p19^{ARF}$, which, by blocking the action of the p53(TP53)-destroying Mdm2, stops excessive oncogene-driven proliferation by stabilizing the cycle-stopping, apoptogenic p53(TP53).[202a] Therefore, these cells would tend to proliferate excessively because they cannot effectively restrain the $G_1$-driving Cdk4·cyclin D1/D2 protein kinases, which causes them to resist shifting into the differentiation mode. However, $p16^{INK\ 4a}$ mutations have rarely been observed in colorectal cancers.[145,203,204] This rarity of disabled mutant $p16^{INK\ 4a}$/$p19^{ARF}$ genes in colorectal cancers is unusual in view of the fact that their frequency is second only to that of p53(TP53). Are colorectal cancer cells really so different from others? The exciting answer is NO! One of the results of the surge of DNA-MTase (DNA methyltransferase) activity that starts along with the loss of APC and the appearance of ACFs is the permanent epimutational silencing of the otherwise normal $p16^{INK\ 4a}$ gene in the cells of 30–40% of colon tumors by the hypermethylation of the CpG islands in the gene's promoter.[145,147]

As in skin carcinogenesis, a striking feature of colon carcinogenesis is a loss of the cells' responsiveness to $Ca^{2+}$.[25,26,41,49,205] Cells in the very earliest stages of carcinogenesis still express the E-cadherin tethers and are still responsive to $Ca^{2+}$. Indeed, at this stage ACF formation in carcinogen (azoxymethane)-treated rats can still be reduced by feeding the animals a diet containing either a suboptimal or superoptimal amount of calcium.[43] Also in humans, the abnormally expanded proliferation zone in the colon crypts of relatives of familial colon cancer patients shrinks back to the lower crypt region when the diet is supplemented with $Ca^{2+}$.[42] However, there are indications of things to come in the $Apc^{-/-}$ familial adenomatous polyposis patients. Incubation in a medium containing 2.2–5.0 mM $Ca^{2+}$ reduces the abnormal proliferation of colon cells taken from 13 out of 14 persons *at risk* of developing colon cancer (because of previous colonic tumors or familial association), but it reduces the growth of colon cells from only 3 out of 10 persons

*actually with* familial polyposis.[206] In other words, the colon cells of those persons with familial polyposis had already lost their $Ca^{2+}$-dependent proliferative control mechanism.

As we learned above, there is a striking *intracellular* $Ca^{2+}$ gradient in the normal colon crypts of humans and mice.[57] This gradient collapses *before* the appearance of tumors in carcinogen (e.g., 1,2-dimethylhydrazine)-treated mice.[207] Moreover, the gradient was found by Brenner et al.[208] to have collapsed even in the crypts from the seemingly normal regions of the mucosa of carcinogen-treated mice with colon tumors, but it had not collapsed in the crypts of the carcinogen-treated mice that did not eventually develop tumors. Thus, the intracellular $Ca^{2+}$ concentration ranged between 212 ± 51 and 228 ± 47 nM in the crypts from normal mucosal regions of tumor-bearing colons, but the intracellular $Ca^{2+}$ concentration was a normal 179 ± 29 nM at the base and 297 ± 62 nM at the mouth of crypts from the colons of mice that had not developed tumors. Therefore, the initiator of tumor development seems to disable the cryptal $Ca^{2+}$ gradient maker mechanism, but we do not know whether there is also a loss of $Ca^{2+}$'s ability to affect proliferation.

The loss of functional APC and the initial loss of responsiveness to $Ca^{2+}$ are associated with a radical shift in the cellular response to a surge of PKC activity specifically that of the overexpressed PKC-βII.[186a] PKC stimulators, such as long-chain diacylglycerols (particularly those with $C_{18:1}$ oleic acid residues), the diacylglycerol-like TPA, and secondary bile acids, inhibit the proliferation of normal colon epithelial cells, but do not affect, or actually *stimulate*, the proliferation of initiated colon cells starting with the $Apc^{-/-}$ familial adenomatous polyposis cells.[209,210] (However, it must be noted that *over-expressed* PKC-β1 inhibits the proliferation of some colon cancer cells.[211]) The reasons for this dramatic conversion of PKC activity from proliferative inhibitor to stimulator at the onset of carcinogenesis are the overexpression of PKC-βII and the downregulation of PKCs α and βI, the ability of PKC-βII stimulated by TPA or the other agents to trigger β-catenin accumulation and thus proliferation and to stimulate another protein kinase that catalyzes the phosphorylation of the Tyr residues of a 63-kDa protein in premalignant and malignant colon cells but *not* in normal cells or those colon carcinoma cells that are still induced to differentiate by PKCs.[186a,212] This other protein kinase is probably c-Src, which is known to be activated early in carcinogenesis, can be stimulated by membrane-associated PKCs, and when activated, decreases the differentiation-promoting $Ca^{2+}$·E-cadherin-mediated cell–cell tethering.[106,213–215] Therefore, the PKCs-stimulating tumor promoters may stimulate the proliferation and expansion of the initiated $Apc^{-/-}$colon cells by stimulating β-catenin buildup and overcoming the $Ca^{2+}$·E-cadherin-mediated suppression of their proliferation by contacting neighboring normal cells just as they overcome the suppression of the expansion of initiated keratinocytes into papillomas by contacting normal keratinocytes.[216–218]

Another important factor in the growing unresponsiveness of progressive generations of initiated colon cells to differentiation signals may be the overexpression of the proliferation-related, cell cycle-driving PKAI with respect to the differentiation-promoting PKAII.[219,220] Indeed, Bradbury et al.[219,220] have found that human colon cancer cells, especially those from poor histological grade tumors, have no detectable RII subunits, while "normal" cells from tumor-adjacent and distant mucosa have both RI and RII subunits. The importance of this declining expression of the differentiation-driving

PKAII is indicated by the ability of RIα-specific antisense oligodeoxynucleotides to suppress, without cytotoxicity, the anchorage-independent proliferation of LS-174T human colon cancer cells in vitro.[221] Moreover, it is further indicated by the ability of subcutaneously injected antisense oligodeoxynucleotides to reduce the growth of these LS-147T tumor cells by as much as 50% in athymic nude mice.[221] Overexpressing RIIβ, but not a disabled RIIβ, in these cells almost completely suppressed PKAI and reduced cell proliferation in monolayer cultures and suppressed their abilities to proliferate in suspension and in a serum-free medium.[222] Thus, the regulatory subunits of the PKAs are targets for a gene-therapeutic strategy to knock colon cancer cells out of the RI-upregulating proliferation mode and into the RII-upregulating differentiation mode.[223]

In about 50–60% of dysplastic ACFs (adenomas), there are $Apc^{-/-}$ cells which also had previously started making a mutant, constitutively signaling, hyperactive Ha-Ras, or much more commonly a Ki-Ras protein with a mutated codon-12 or -13.[149,155,163,170–172,193] These hyperactive Ras proteins can block the differentiation of epithelial cells, are mitogenic, and eliminate the cell's need for external $Ca^{2+}$ to proliferate.[224,225] They drive the proliferogenic MEK/MAP kinase cascade by activating the Raf-1 protein kinase (see Chap. 2). As we learned in the previous chapter, activated Ras proteins such as Ha-Ras can stimulate PtdIns-3K, which in turn stimulates the ILK and PKB protein kinases that suppress E-cadherin expression, inhibit GSK-3β, and thus keep the cycle-driving β-catenin flowing into the nucleus.[12,67] It would also appear that Ras-activated Raf-1 kinase phosphorylates BAD protein, which releases it from inactive BAD·Bcl-2 complexes and at the same time frees Bcl-2 to dimerize with itself or heterodimerize with Bax protein to promote cell survival.[9,226] But Ki-Ras is rather special. The mutant Ki-Ras, *unlike* a mutant Ha-Ras, can prevent the colon cells' apicobasal polarization and upregulate CEA (carcinoembryonic antigen), which prevents the formation of normal, laterally adhering polarized colon cells.[227] Thus, the hyperactive Ki-Ras mutant would disrupt differentiation and collaborate with an abnormally expressed Bcl-2 protein and a surging β-catenin to further enhance the abilities of adenoma and carcinoma cells to continue proliferating even in the killing zone of the crypt and on the mucosal surface despite the increasingly intense, and normally apoptogenic, signaling from the CaRs. Ultimately, a hyper-signaling Ki-Ras mutant, an upregulated Bcl-2, a disabled or lost β-catenin-destroying APC, and an increasingly overexpressed PKAI combine to stamp out traces of normal differentiation and drive the adenomatous development to the threshold of malignancy.

As the mass of competing mutant clones progresses from polyp to premalignant adenoma, there is an increasing loss of responsiveness to $Ca^{2+}$. The cells still need external $Ca^{2+}$ to proliferate, but even this will be lost with the transition from adenoma to carcinoma. However, the proliferation-stopping, differentiation-triggering $Ca^{2+}$-switch no longer works. Thus, for example, the supernormal proliferative activity of primary human colon adenoma cells in the presence of 0.05–0.1 mM $Ca^{2+}$ can be increased even further by raising the $Ca^{2+}$ concentration to a high physiological level such as 2.2 mM, which would stop normal colon cells proliferating.[39] Therefore, the cells of mutant clones can proliferate, indeed be induced to proliferate more rapidly, when confronted by the high $Ca^{2+}$ concentration in the fecal stream.

Loading the diet with $Ca^{2+}$ (e.g., with 2 g/day as calcium carbonate or phosphate) reduces the compensatory, tumor-promoting hyperproliferation of colon crypt cells caused by the destruction of cells on the flat mucosal surface by cytolytic surfactant

secondary bile acids and long-chain fatty acids in the fecal water of persons consuming a typical high-fat, low-fiber, Western diet.[48,49] Since it prevents the appearance of clones with mutant Ras proteins and reduces the size and number of carcinogen-induced tumors in rats by forming inactive complexes with the cytolytic secondary bile acids and long-chain fatty acids in the fecal water, it should reduce the risk of colorectal cancer in humans.[48,49,228,229] Indeed, $Ca^{2+}$ reduces the hyperproliferative activity in the colons of high-risk humans and normal mice mainly by promoting apoptosis and thus shrinking the cryptal proliferative zone rather than inhibiting proliferation.[44–49,229a] However, a key question remains as to how accessible the proliferative zones in the depths of the crypts are to $Ca^{2+}$ in the fecal water. Loading the crypts with $Ca^{2+}$ would directly reduce the proliferation and promote the differentiation of normal cells as well as still phenotypically but early initiated cells. However, this loading would act like the tumor-promoting TPA by suppressing normal cells without affecting or even stimulating the proliferation of initiated cells.[49] This possibility is supported by the observation that loading the diet of rats treated with the carcinogen azoxymethane with $Ca^{2+}$ when carcinogenesis was in full swing (as indicated by extensive ACF formation) actually greatly *increased* the number of tumors located 12–16 cm from the rectal end.[49] It is also supported by reports that dietary $Ca^{2+}$ supplementation *stimulates* the proliferation of epithelial cells in the colons of patients with adenomatous polyps.[230,231]

There is likely to be another serious drawback to loading colons containing initiated cells with $Ca^{2+}$ above a certain concentration. Cells in the early post-initiation stages are probably proliferatively restrained by things flowing into them from their normal neighbors through their still functional gap junctions.[18] However, gap junctions are designed to be slammed shut by $Ca^{2+}$.[18] This is meant to prevent the draining of components from the cellular network through a damaged dying cell.[18] $Ca^{2+}$ was chosen as the damage detector and drain plugger simply because its accumulation is a universal consequence of cell injury. The closing of gap junctions by supra-optimal $Ca^{2+}$ loading would release the initiated colon cells from the restraining influences of their normal neighbors.

Yet another drawback of dietary $Ca^{2+}$ loading is of particular concern to men. There is a report that $Ca^{2+}$ from food sources and dietary supplements increases the risk of advanced prostate cancer.[232]

## *The end of the road — a lucky winning clone and colon carcinoma*

As the tumor promoters in the fecal stream and the increasingly endogenously unrestrained CDKs relentlessly drive the proliferation of mutating clones, a lucky winner of the mutation lottery will emerge with all the mutations (Greaves's "Full House")[232a] it needs to escape from advanced adenoma to the ultimate freedom of carcinoma and be resistant to apoptosis.[232b] Adenoma cells had increased their expression of the Bcl-2 protector protein, but during the transition to carcinoma, Bcl-2 is downregulated and its anti-apoptogenic relative Bcl-w is upregulated to maintain the protection.[1,233] Another anti-apoptosis change, which occurs in about 50–75% of large adenomas on the threshold of adenocarcinoma, is a loss-of-function mutation of the p53(TP53) gene on the short (p) arm of human chromosome 17 (17p13 to be precise).[123] The disabled p53(TP53) mutant protein has a prion-like "infectiousness": it can associate with the product of the normal allele and cause the normal protein to reconfigure itself to the inactive mutant form.[234] In

other words, the $G_1$/S checkpoint of a p53(TP53)$^{+/-}$ heterozygote is as useless as that of a p53(TP53)$^{-/-}$ homozygote. But eventually cells appear which have also lost their normal allele. The loss of p53(TP53) function coincides with the appearance of carcinoma in the polyp or adenoma. It has eliminated a major safety checkpoint guardian, which when activated by DNA damage would normally stop cells proliferating until the damage is repaired, or failing this, trigger apoptosis.[9,92,95,235] Therefore, the loss of functional p53(TP53) enables the survival and expansion of dangerous criminal clones. Obviously, the combination of a defective mismatch repair mechanism due to mutation or methylation of the hMLH1 gene promoter, such as is found in some sporadic colon carcinomas,[196] and the loss of p53(TP53) would give a very strong push to the accumulation of mutations and epimutations, which are responsible for the emergence of carcinoma clones. And the upregulation of Bcl-w would help protect them from the ravages of potent chemotherapeutic agents or the dangers of metastasis.

The ultimate appearance of carcinoma (malignant) cells is tightly coupled to the loss of TGF-β signaling, which is most often due to the disabling mutation of the Type II receptor, but can also be due to the loss of functional Smad proteins (Smad 2 or Smad 4) that mediate the proliferation-stopping response of normal colon cells to TGF-βs.[99a] Also, in about 70–90% of colon carcinoma cells, but not adenoma cells, the DCC gene (the smad 4 (DPC4) gene's neighbor on the short arm of human chromosome 18)[235a] is disabled. This is an important step toward malignancy because the DCC receptor, a differentiation promoter and migration guide, triggers apoptosis if a normal cell, in a misguided attempt to escape from its niche, should pull it away from its laminin-like netrin-1 ligand in the migration trackway.[126–131] In other words, DCC signaling is part of a tumor-suppressing, fail-safe anti-metastasis mechanism, without which the cell can safely lift anchor and metastasize to distant places and ultimately kill its owner.[131,193,235a]

Up to now, the roiling mass of struggling but still tightly tethered clones has been safely confined to a "carcinoma in situ." Eventually, breakaway cells will emerge which cannot make the $Ca^{2+}$-E-cadherins with which their progenitors tied themselves to their neighbors and sequestered the cycle-promoting, differentiation-restraining β-catenin.[81,236–241] But how can this be, since the emerging malignant mutants' E-cadherin genes seem to be perfectly normal?[146] What has happened? As in the case of the loss of $p16^{INK\,4a}$ function, the failure to make the protein appears to be one of the consequences of a rising level of DNA-MTase, which replicably and permanently silences the still normal E-cadherin gene by hypermethylating the cytosines of a CpG island that spans the gene's transcription start site.[81,145,147]

Yet another mutation involved in the enabling of metastasis is an upregulation of the expression of the $Ca^{2+}$-binding S100A4 protein.[242] This is in addition to the earlier replacement of the normal cell's expression of S100A6 isoform IV with the expression of S100A6 isoforms I and III.[243]

With these several mutations and epimutations and their strong Bcl-w shield, the carcinoma cells have completed the divorce from control by external $Ca^{2+}$ that began when their carcinogen-damaged ancestor lost its functional APC. The extent of this divorce is indicated by the fact that while the cells in the early hyperplastic stage up to the well-differentiated adenocarcinoma stage still express CaRs, these receptors, and with them the cells' responsiveness to proliferation-restraining signals from external $Ca^{2+}$, vanish in the later stages.[63a,b]

As we have learned, the loss of functional APC results in the over-expression and a buildup in the nucleus of the cycle-driving β-catenin. However, it seems that β-catenin is most strongly expressed by the cells on the tumor's invasion front, while cells in the core of the tumor have little or no β-catenin in their nuclei, but have it on their cell membranes as do normal colon cells.[244] This raises the possibility that normal cells somehow tell the cells in the oncoming tumor invasion front to load their nuclei with β-catenin.[244]

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# 5
# Calcium and Neurons

We have seen $Ca^{2+}$ and its assistants orchestrating key parts of the cell cycle and terminal differentiation or diffpoptosis, that ingenious amalgam of differentiation and apoptosis, of keratinocytes and colon cells. Now we travel from the gut to the brain (but we must not forget that the gut has its own large 'brain' [Gershon's second brain][1]) where $Ca^{2+}$ truly reigns supreme in a role that has expanded from its first job of being the ionic eyes and ears of the first eukaryotic cells to being one of the central figures in the sensory systems of multicellular animals.

Although not experimentally confirmed, we can be sure that $Ca^{2+}$ spikes and oscillations and cresting and crashing waves of CDKs drive the proliferation of the multipotent ependymal progenitor cells in the embryonic brain and the persistent subventricular generative zones of the lateral ventricle and hippocampal dentate gyrus of the adult brain that provide neurons and glial cells for dentate gyrus and olfactory bulb, just as they drive the cycling of basal keratinocytes and colon crypt cells.[2–8] Here too we find the CaRs with as yet undefined functions, although they may function in modulating cation channel-dependent establishment of LTP (long-term potentiation) in hippocampal neurons and help drive the proliferation of glial cells and neuroblasts.[8a,9,10] But there is something special about mature neurons. They are proliferatively disabled like post-mitotic keratinocytes and colon cells, maybe because of unlicensed replication origins or the locking up of the genes for key parts of replication factories. Unlike the very short-lived keratinocytes and colon cells, they are very long-lived and can still grow. Through activity-driven growth without proliferation, called *plasticity*, they continuously shape and reshape the brain to produce a structural/functional configuration as characteristic of the individual as the fingerprints.

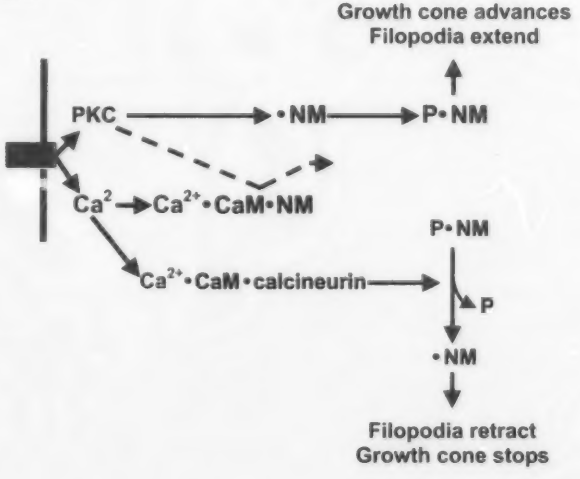
To build the basic 'starting' brain, neurons must climb along supporting glial cells or other neurons and send out processes (neurites) to synaptically contact other neurotropin-emitting neurons and peripheral target cells. $Ca^{2+}$ and its now familiar assistants such as CaM and CaMKII, and undoubtedly the CaRs, are at the helms of the axonal growth cones. Indeed, $Ca^{2+}$ transients modulate growth and the expression of differentiation-related genes according to their frequency and distribution.[10a] The advancing tip of a neurite, the autonomously driven growth cone,[11,11a] moves along a track marked by a set of matrix components or 'bricks' such as the netrins, which it grabs with the DCC (deleted in colon cancer) receptors that we met in the previous chapter.[12–15] Some of the matrix guideposts say 'go forward!' while others say 'stop and turn!,' depending on the kinds of signals from the various guidepost receptors and the gradient of diffusible factors emanating from the target.[13] Thus, for example, the repellant MAG (myelin-associated glycoprotein) and the attractant netrin-1 attract if the cellular cyclic AMP level be high, but repel if the level be low.[16] The growth cone has a large finger(s), a

filopodium with a bundle of microfilaments, that grabs the trackway's 'bricks' with its receptors and pulls the process along the trackway.

$Ca^{2+}$'s role in neurite production and growth cone navigation depends on the location, frequency, and amplitude of $Ca^{2+}$ spikes and waves.[10a,16–18] It seems low-frequency $Ca^{2+}$ surges drive neurite growth, but when a rapidly advancing growth cone with a few or no $Ca^{2+}$ transients reaches a turning point on the trackway it slams its brakes on with a burst of $Ca^{2+}$ transients (from both the smooth endoplasmic reticulum and probably voltage-gated membrane channels at the extreme leading edge) that stop migration by causing the dispersal of the finger's bundle of microfilaments, the withdrawal of the finger, the expansion of the cone, and the generation of multiple protrusions. The frequency of the $Ca^{2+}$ surges then drops. Depending on the position of the peripheral→central $Ca^{2+}$ gradient in the growth cone, a new filopodium(-ia) protrudes and the cone sets off again in a new direction with a much reduced frequency of $Ca^{2+}$ transients.[10a,11a,16a,19]

It seems likely that $Ca^{2+}$ somehow works through a molecular switch, a growth-associated protein known as neuromodulin or GAP-43, the overexpression of which enhances memory in mice.[11a,b] This switch is concentrated on the inner surface of the plasma membranes of axons and growth cones and the growth cones' cortical cytoskeleton where it can increase actin filament length, hence filopodium/lamellipodium protrusion and growth cone migration.[11a] The $Ca^{2+}$·CaM produced by a $Ca^{2+}$ transient binds to the switch's IQ (isoleucine-glutamine) site that also contains $Ser^{41}$, which

**Fig. 5.1.** A neuromodulin (NM) switch with which $Ca^{2+}$ and PKC might control the movements of axonal growth cones. An activated navigation factor receptor sends a $Ca^{2+}$ signal that produces $Ca^{2+}$·CaM that binds to NM's IQ motif (•), which also contains PKC's target residue, $Ser^{41}$. When the $Ca^{2+}$ signals are infrequent and $Ca^{2+}$·CaM production is low, PKC can get at NM's IQ domain to make the phospho-NM (P•NM) needed for filopodial production and growth cone advance. However, if $Ca^{2+}$ signaling and $Ca^{2+}$·CaM production exceed a critical level, $Ca^{2+}$·CaM will block the IQ domain and prevent PKC from phosphorylating NM. The $Ca^{2+}$·CaM also stimulates the phosphatase calcineurin that dephosphorylates existing phospho-NM. The conflicting convergence of $Ca^{2+}$·CaM and PKC on NM's IQ domain and the $Ca^{2+}$·CaM-stimulated calcineurin thus combine to cause the filopodia to retract and stop the growth cone advance.



happens to be the target for phosphorylation by PKC (Fig. 5.1). This is how $Ca^{2+}$ controls the switch, because it is PKC- phosphorylated neuromodulin that is required for the formation of filopodia and the moving of the growth cone toward its target.[11a] The subcritical amount of $Ca^{2+}$·CaM produced by a low-frequency of well-spaced $Ca^{2+}$ signals from the surface receptors for a growth factor or a trackway component would not prevent activated, membrane-associated PKC from phosphorylating neuromodulin's $Ser^{41}$. The PKC-phosphorylated neuromodulin then promotes filopodia extension and growth cone migration. However, if the frequency of $Ca^{2+}$ transients and the resulting $Ca^{2+}$·CaM production should exceed a critical level, it would promptly bind to the IQ site before the more slowly responding PKCs could get to it and phosphorylate $Ser^{41}$. At the same time, the $Ca^{2+}$·CaM will also stimulate the phosphatase calcineurin, which would dephosphorylate existing phospho-neuromodulin. The conflicting convergence of $Ca^{2+}$·CaM and PKC on the switch's IQ domain and the surge of $Ca^{2+}$·CaM·calcineurin activity combine to cause filopodia to retract and stop the growth cone's advance.

In the last two chapters, we learned that PTHrP plays a key role in the differentiation of cartilage, colon, and skin by delaying the final apoptotic demise of the differentiating cells. It is beginning to appear that the peptide also prevents apoptosis in at least some neurons. Depolarizing cerebellar granule cells with excessive external $K^+$ or activating *N*-methyl-D-aspartate (NMDA) receptor/channels results in the opening of L-type $Ca^{2+}$ channels and a surge of $Ca^{2+}$·CaM, which stimulates the expression of PTHrP that enhances the survival of the neurons making and secreting it.[20,21] The autocrine/paracrine PTHrP does this by stimulating the producer cells to express the Bcl-2 anti-apoptotic protector protein.[22] However, PTHrP does something else — it prevents an excessive or prolonged $Ca^{2+}$ surge through L-type $Ca^{2+}$ channels in kainic acid-treated granule cells from killing the cells through a cyclic AMP/PKA-induced phosphorylation and inactivation of the channels.[20] Thus, it seems worth speculating that the intense signaling of neurons and their neurites when interacting normally with the various 'bricks' in their trackways or from excessively sustained glutamate receptor activation and depolarization following injury may produce the $Ca^{2+}$·CaM needed to produce, or at least try to produce, enough PTHrP to protect themselves from excitotoxic and apoptotic death.

Eventually, successful neurons establish synaptic contact with their targets. Complex, cross-talking systems are set up to cause the release of neurotransmitters and drive signal transmission and the memory of transmitted signals. Now we must look at this wonderful synaptic world from the viewpoint of $Ca^{2+}$.

The growing axons with their probing growth cones eventually meet and form connections — synapses — with other neurons to form a neural net. These synaptic connections can be strengthened or weakened — a property known as "plasticity."[23–25] Plasticity is an essential feature of synaptic transmission, and depending on the nature or pattern of the preceding signal, the synaptic activity can either be enhanced or suppressed, a phenomenon called "activity-dependent synaptic plasticity" or ADSP.[24,26–28] ADSP is considered to be extremely important for many neuronal functions including learning and memory. It is a fundamental property of various types of synapse in both vertebrate and invertebrate central and peripheral nervous systems.[29–33] Several forms of plasticity at both presynaptic and postsynaptic sites have been described which briefly or prolongedly enhance or suppress synaptic transmission.[26–30] This enhancement or suppression might last from milliseconds to several days.[34] Studies over the years have clearly

demonstrated that all forms of plasticity are linked to a rise in the intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$). Although it is not quite clear as to how something as seemingly simple as a change in the $[Ca^{2+}]_i$ could induce such diverse and specific forms of plasticity, it is becoming apparent that the origin of the $Ca^{2+}$ and where in the cell it surges and crashes determine the kind of plasticity.[24,26,34] Moreover, the amplitude and the frequency of individual $Ca^{2+}$ signals, which occur in the spatial and the temporal domains, respectively, also contribute to the diversity and the specificity of the plasticity.[24,26,34]

## Intercellular $Ca^{2+}$ waves and signaling networks

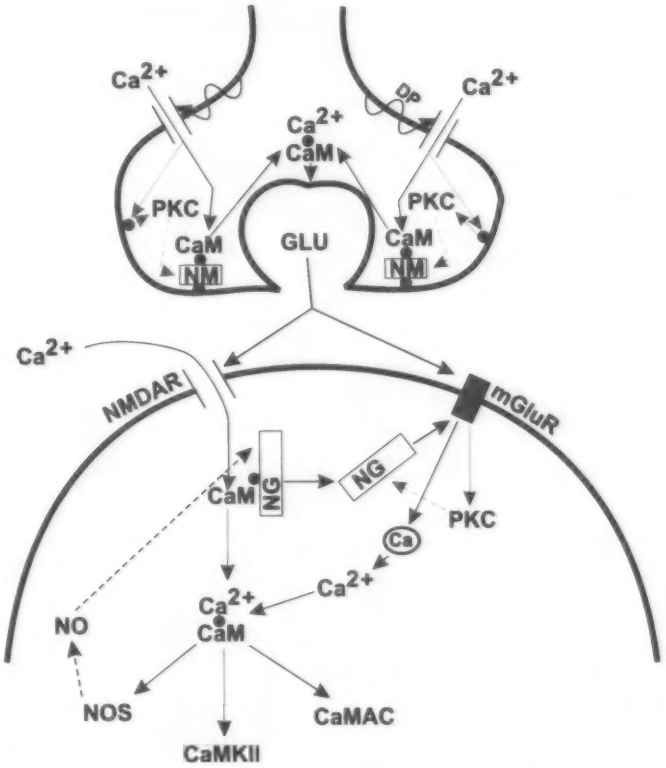
Before going down to the individual neuron, we must look at the whole picture of the flow of $Ca^{2+}$ signals throughout the brain.[34a] Neurons are surrounded by, and talk to, glial cells — there is a reciprocal exchanging of information in the forms of ions and the neurotransmitter glutamate.[34b,c] $K^+$ and glutamate released from neurons can reach adjacent glial cells where they activate $Ca^{2+}$ channels and receptors, which results in receptor-activated PLC (phospholipase-C) activity that generates $InsP_3$ (inositol-1,4,5-trisphosphate) and an $InsP_3$-induced release of $Ca^{2+}$ from internal stores, which produces $Ca^{2+}$·CaM and stimulates various enzymes such as type 1 adenylyl cyclase, protein kinases, and phosphatases. Each glial cell is in turn connected to a network of other glial cells and neurons by as many as 30 000 gap junctions through which $InsP_3$ can flow and trigger the release of $Ca^{2+}$ from more internal stores. The passage of the $Ca^{2+}$ wave with its wake of $Ca^{2+}$·CaM complexes through the glial cell toward the gap junctional passageways to other glial cells or neurons is amplified at "way stations" along the endoplasmic reticulum consisting of clusters of associated mitochrondria.[34a] The mitochondria-amplified $Ca^{2+}$ waves also stimulate NO synthase that makes the NO that causes the vascular dilation needed to increase the blood supply to active neurons, which is an indicator used in functional brain scanning.

## Presynaptic $Ca^{2+}$ signaling and short-term plasticity

Although long-term synaptic plasticity has been the main focus of research because of its importance in learning and memory, recent research has focussed on short-term synaptic enhancement because of its importance in information processing, a principal function of neurons.[26,34] In most synapses, the action potential arriving at the nerve terminal triggers a transient increase in $[Ca^{2+}]_i$, which can induce the expression of various forms of short-term synaptic plasticity at the presynaptic site (Fig. 5.2).[34] The major presynaptic entry points of $Ca^{2+}$ are N-type and P/Q-type voltage-sensitive $Ca^{2+}$ channels (VSCCs), which cluster around the transmitter release sites on the axon terminals.[35–36a] The transient surge of $Ca^{2+}$ through these channels is responsible for the release of neurotransmitters, which is seen as changes in the postsynaptic or junctional potential. If the $[Ca^{2+}]_i$ is still elevated when the next action potential arrives, the preexisting calcium ions combine with the incoming ions to produce a longer-lasting $Ca^{2+}$ surge. This progressive, activity-driven $Ca^{2+}$ buildup in the presynaptic terminal is believed to be the basis of the short-term enhancement (STE) of synaptic transmission.

It was initially thought that STE is a single event,[34] but it is now clear that it consists of three main phases, facilitation (lasting less than a second), augmentation (lasting

**Fig. 5.2.** How CaM and PKCs may affect the $Ca^{2+}$-induced release of a neuron's axonal neuromodulin-bound and its dendritic neurogranin-bound CaM stores and the release of the neurotransmitter glutamate into the synaptic space. The emptying of these CaM stores provides the CaM for making the $Ca^{2+}$·CaM needed for the presynaptic release of glutamate and the postsynaptic stimulation of various $Ca^{2+}$·CaM-dependent enzymes. The chain of events, started by a surge of $Ca^{2+}$ through depolarization(∿)-opened VSCCs (voltage-sensitive $Ca^{2+}$ channels) in the axon terminus, which lead to the release of CaM stores, glutamate secretion, stimulation of postsynaptic NMDA and mGluR receptors, and the postsynaptic accumulation of $Ca^{2+}$·CaM are described in the text. CaMAC, $Ca^{2+}$·CaM-dependent adenylyl cyclase; CaMKII, $Ca^{2+}$·CaM-dependent protein kinase II; GLU, glutamic acid; mGluR, metabotropic glutamate receptor; NG, neurogranin; NM, neuromodulin; NMDAR, NMDA glutamate receptor; NO, nitric oxide; NOS, nitric oxide synthase; DP, depolarization. Solid lines, stimulation; broken lines, inhibition/blockage.




several seconds), and post-tetanic potentiation (PTP, lasting a few minutes).[37,38] Facilitation, in turn, may have a fast-decaying (F1) and slow-decaying (F2) component.[29,31,34] All these phases depend on a rise in $[Ca^{2+}]_i$. When an action potential reaches the nerve terminal and opens VSCCs, the $Ca^{2+}$ streaming through these channels creates microdomains or ephemeral hot spots where the $[Ca^{2+}]_i$ could be as high as 100 μM,[39–41] and it is at these hot spots where $Ca^{2+}$ drives transmitter release by binding to low-affinity sites.[42] The length of time the VSCCs are open is directly related to the duration of the action potential. When the action potential subsides, the VSCCs close, $Ca^{2+}$ flow stops, and

the $Ca^{2+}$ hot spots quickly vanish as their $Ca^{2+}$ diffuses into the core of the nerve terminal where it is sequestered by intracellular buffers.[26,34,43] However, a small amount of $Ca^{2+}$ remains free to modestly increase the residual [$Ca^{2+}$]. Katz and Miledi[44] were the first to propose a "residual $Ca^{2+}$" hypothesis to explain facilitation of neurotransmission at the neuromuscular junction in frogs. In their experiments, removing extracellular $Ca^{2+}$ during the initial stimulation (conditioning) eliminated the facilitation of transmitter release during a subsequent "test" stimulation when $Ca^{2+}$ had been put back in the medium. Therefore, they proposed that a certain fraction of the $Ca^{2+}$ that surged into the presynaptic terminal during the initial stimulation stays around in the terminal to supplement the $Ca^{2+}$ surge triggered by the next action potential and to enhance transmitter release. Thus, each action potential would be expected to, and in fact does, add to the residual $Ca^{2+}$ pool. For example, it has been estimated that in crayfish terminals the $Ca^{2+}$ load from each action potential increases the amount of $Ca^{2+}$ in the residual pool by 10 nM.[45] In small granule cell-Purkinje cell synaptic terminals, a single action potential can increase the residual $Ca^{2+}$ by as much as 200–300 nM, which subsequently drops with a time constant of 100–200 ms.[46,47] When this terminal is bombarded with a high-frequency train of action potentials, the residual $Ca^{2+}$ level can rise as high as 1000 nM and then slowly decline with a time constant of several seconds to minutes.[48–50]

It is now well accepted that the residual $Ca^{2+}$ affects all three phases of presynaptic enhancement: facilitation, augmentation, as well as the potentiation.[34] But how does the level of residual $Ca^{2+}$ affect these enhancement phases each with its own decay kinetics? To understand this, one must consider the spatiotemporal dynamics of $Ca^{2+}$ distribution.[26,34,38] As we learned above, there are ephemeral high-$Ca^{2+}$ hot spots clustered around, or very near, the channels through which the ion flows. These hot spots are very different from the slow, low-concentration, and diffusely spread residual $Ca^{2+}$ in the core of the terminal. Therefore, the $Ca^{2+}$ binding sites of these two compartments are exposed to very different levels of $Ca^{2+}$. If the binding sites are directly involved in exocytosis (transmitter release), then their location determines how fast the enhancement of transmitter release occurs. Thus, the $Ca^{2+}$ in the hot spots can more rapidly trigger transmitter release than in the core of the terminal. The hot-spot $Ca^{2+}$ transients may contribute to the facilitation (F1 and F2) phase of the enhancement, while the residual core $Ca^{2+}$ may contribute to longer lasting augmentation and potentiation phases of synaptic enhancement.[34,44,48,49,51,52]

While residual $Ca^{2+}$ theory generally holds, it is not clear whether bound or free $Ca^{2+}$ plays a critical role in establishing STE. Some studies indicate that the residual unbound $Ca^{2+}$ in the hot spots on or near the sites directly linked to exocytosis is responsible for F1 and F2 components of facilitation.[44,51,52] However, in some other studies $Ca^{2+}$ chelators, at concentrations high enough to block transmitter release, did not affect facilitation, which suggests only a minimal role of residual unbound $Ca^{2+}$ in this process.[53] It is likely that $Ca^{2+}$, bound to targets that are either directly or indirectly linked to transmitter release, might play a role. It is known that the exocytosis-target sites must bind at least 4 $Ca^{2+}$ ions to trigger transmitter release.[41,54] If $Ca^{2+}$ stays bound to one or more of these sites after the passing of an action potential, it would facilitate the triggering of transmitter release by a subsequent action potential when the remaining sites are occupied by the incoming $Ca^{2+}$.[34]

Experimental results have suggested that different phases of synaptic enhancement correspond to various time constants of the decay of residual $Ca^{2+}$ and also depend on the action of $Ca^{2+}$ on at least two different sites.[24,26,47–49,55] Thus, the $Ca^{2+}$ bound to low-affinity binding sites in the hot spots near the $Ca^{2+}$ channels rapidly fans out into the core where the induction of the F1 and F2 facilitation phases is believed to occur.[44,51,52] The residual, diffuse $Ca^{2+}$ in the terminal's core drops relatively slowly, and it is here where augmentation and post-tetanic potentiation are thought to occur.[48,49] Generally, the residual $Ca^{2+}$ is pumped out of the cell by ATP-driven $Ca^{2+}$ pumps in the plasma membrane and (or) is sequestered in endoplasmic reticulum, mitochondria, or endoplasmic reticulum–mitochondrial clusters.[34a,43,56,57] Thus, the speed of the removal of residual $Ca^{2+}$, which determines the duration of the $[Ca^{2+}]_i$ transients, is strictly a function of the efficiency with which these $Ca^{2+}$-handling mechanisms operate and which may vary from terminal to terminal. AUG and PTP require a large number presynaptic action potentials for induction.[58,59] The buildup of $Ca^{2+}$ by a rapid train of APs will result in a rapid loading of mitochondria, which then slowly release their $Ca^{2+}$.[34a,50,60–62] Augmentation subsides as the unbound $Ca^{2+}$ in the terminal core is pumped out of the cell. However, the slow extrusion of $Ca^{2+}$ from the mitochondria causes a brief surge of core $Ca^{2+}$, which is probably responsible for the potentiation before it is ejected from the neuron by the membrane $Ca^{2+}$ pumps.[50] Indeed, blocking mitochondrial $Ca^{2+}$ release abolishes PTP.[50] Although the uptake and release of $Ca^{2+}$ by the endoplasmic reticulum do not seem to play a role in presynaptic activity in some synapses,[50] both mitochondria and endoplasmic reticulum seem to play a role at other synapses.[63,64]

While the induction of presynaptic STE appears to be largely due to $Ca^{2+}$ entry through the VSCCs during an action potential, recent studies indicate that other sources of $Ca^{2+}$ may also contribute to this process. A $Na^+$-dependent increase in $[Ca^{2+}]_i$ has been implicated in short-term enhancement of synaptic transmission, particularly PTP.[34,48,65] This $Ca^{2+}$ surge could be due to $Na^+$-induced increase in the release of intracellular $Ca^{2+}$ or the blockade of $Ca^{2+}$ extrusion by the $Na^+/Ca^{2+}$ exchanger.[34] Both ionotropic and metabotropic glutamate receptors, which have also been found in the presynaptic sites, appear to be involved in the establishment STE.[66–68] It is suggested that the facilitation of transmission occurs when metabotropic glutamate receptors activate $Ca^{2+}$-dependent release of $Ca^{2+}$ from internal stores.[68,69] Furthermore, presynaptic $Ca^{2+}$ channel activity can be modulated by activated G-protein coupled metabotropic receptors,[70] and $Ca^{2+}$ currents affected by these receptors can modulate presynaptic transmitter release.[68,69] Similarly, ionotropic receptors such as the NMDA, AMPA, and kainate receptors, may alter the level of presynaptic $Ca^{2+}$ before the arrival of an action potential and thereby contribute to enhanced presynaptic transmission.[66,67,67a,67b] Such a mechanism has been proposed for nicotinic receptors at the hippocampal mossy fiber terminal[71] and for glutamate receptors at the lamprey reticulospinal axons.[66] The action of these receptors could be direct by allowing $Ca^{2+}$ through their channels or indirect by depolarizing the terminal to activate presynaptic VSCCs.[66–71] Although the site of STE is predominantly presynaptic, recent studies in *Aplysia* neurons have indicated that preventing a postsynaptic $Ca^{2+}$ rise or inducing hyperpolarization can actually block the induction of STE,[72–74] suggesting that postsynaptic cell may also contribute to STE, at least during the initiation stage.

## Postsynaptic $Ca^{2+}$ signaling and long-term plasticity

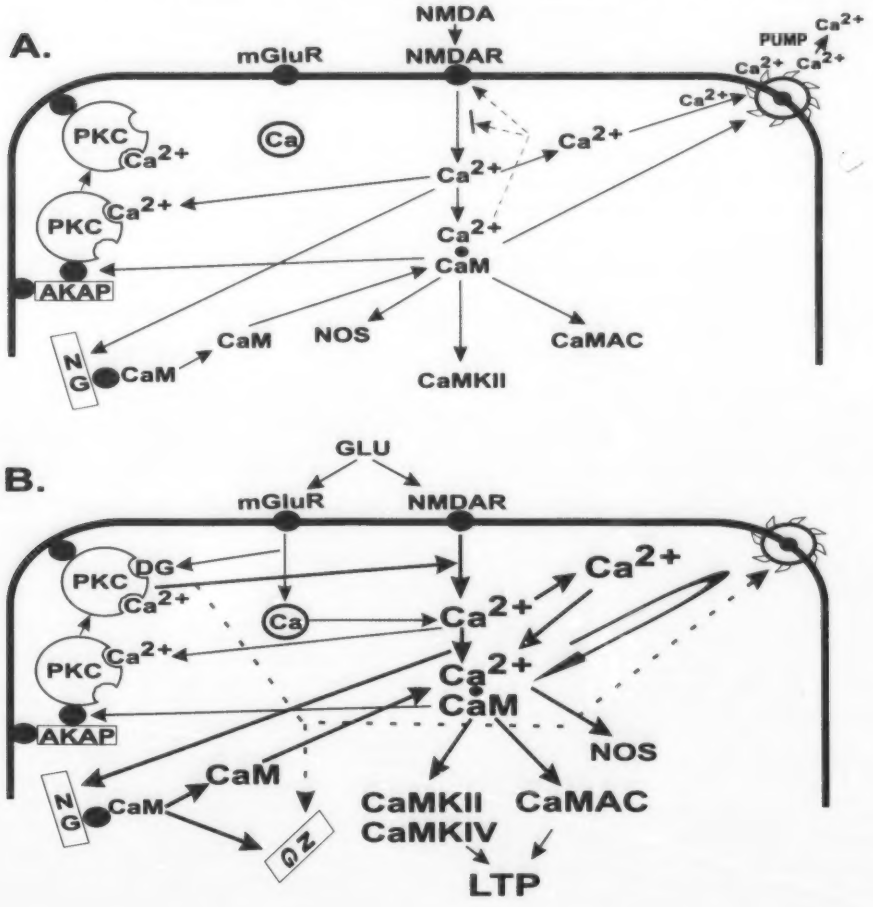
It is clear from the preceding section that presynaptic $Ca^{2+}$ plays a pivotal role in synaptic plasticity and the dumping of neurotransmitters out of presynaptic storage vesicles. When these transmitters activate their receptors on postsynaptic membranes, they modulate $Ca^{2+}$ dynamics in the associated postsynaptic dendrites, neuronal soma (body), or axon. As in the presynaptic site, an increase in postsynaptic $[Ca^{2+}]_i$ also gives rise to various forms of plasticity. However, the impact of $Ca^{2+}$ signaling at the postsynaptic site is much more complex than at the presynaptic sites because of the variety of $Ca^{2+}$ sources and the greater number of intracellular compartments and targets.[24,75,76] Unlike presynaptic $Ca^{2+}$, postsynaptic $Ca^{2+}$ generates longer lasting plasticity, the duration of which persists well after the inducing-signal and $Ca^{2+}$ surges have disappeared.[24,77,78] Thus, $Ca^{2+}$ triggers rather than maintains postsynaptic plasticity. This long-lasting effect of $Ca^{2+}$ on postsynaptic plasticity forms the basis of long-term potentiation (LTP) that was first discovered in mammalian hippocampus[79,80] and has subsequently been shown in other brain structures.[81–82*a*] Recent studies[83] indicate that this longer lasting $Ca^{2+}$ signal also induces another form of plasticity, long-term depression (LTD).

How could the same $[Ca^{2+}]_i$-elevating postsynaptic event on one hand strengthen (LTP) and on the other hand weaken (LTD) synaptic transmission in the same neuron? As we will see further on, the amplitude and the spatiotemporal distribution of the $Ca^{2+}$ signals may play a role in this process, with LTD having a lower $Ca^{2+}$-induction threshold than LTP.[26,84–86] Such long-term effects of $Ca^{2+}$ on synaptic plasticity appears to be crucial for learning and memory. Since most of the work on LTP and LTD has employed the excitatory synapses of hippocampal glutamatergic neurons, most of the following discussion is based on these studies. However, it is believed that the mechanisms of LTP and possibly LTD are the same in all the glutamatergic neurons.

While the $Ca^{2+}$ involved in presynaptic plasticity comes into the cell through action-potential-opened VSCCs (Fig. 5.2), there are various postsynaptic sources of $Ca^{2+}$ that may contribute to different forms of plasticity.[26,87] The main sources of postsynaptic $Ca^{2+}$ are the VSCCs, ligand-gated calcium channels (LGCCs) such as the NMDA glutamate receptor channel and internal $Ca^{2+}$ stores, such as endoplasmic reticulum and mitochondria, all of which have unique spatial distributions and properties.[24,34*a*,87–89] Although it is now virtually certain that $Ca^{2+}$ from all these postsynaptic sources either independently or together is involved in some form of synaptic plasticity,[88] the mechanism by which the $Ca^{2+}$ signals are processed and integrated in enhancing/potentiating postsynaptic transmission is not clearly understood.

The major source of $Ca^{2+}$ for the induction of LTP is believed to be the widely distributed postsynaptic glutamate-type NMDA receptor channel (Figs. 5.2 and 5.3).[88–91] Indeed, preventing $Ca^{2+}$ from entering through this channel with selective inhibitors, like APV and MK-801, or the intracellular injection of $Ca^{2+}$ chelators inhibit most forms of LTP and LTD.[92–99] NMDA receptor channels are heavily concentrated in postsynaptic densities often along with AMPA-type glutamate receptor channels.[25,100–102] A unique property of the NMDA receptor channel is its voltage dependence because under resting conditions it is plugged by $Mg^{2+}$, which can only be expelled by a positive voltage shift.[103–105] This means that two things must occur if $Ca^{2+}$ is to flow through an NMDA receptor — the receptor must bind glutamate as it is dumped out of presynaptic vesicles,

**Fig. 5.3.** The influence of conflicting convergences of $Ca^{2+}$·CaM and PKCs on the postsynaptic responses to the stimulation of NMDA receptors only (**A**), or NMDA and mGluR receptors (**B**). When NMDA receptor/channels alone are activated (**A**), the response is brief because the $Ca^{2+}$ flow through the channels is switched off by $Ca^{2+}$·CaM feeding back and binding to the receptors' R1 subunits, and the $Ca^{2+}$ that has entered the cell is expelled when $Ca^{2+}$·CaM stimulates the membrane $Ca^{2+}$ pumps. $Ca^{2+}$·CaM also displaces PKCs from their binding site on AKAP79 in postsynaptic densities. The liberated PKCs bind to the cell membrane, but whether they will be activated depends on the availability of Dgs (diacylglycerols). If mGluR receptors are also activated (**B**), DGs from the PtdIns(4,5)$P_2$ breakdown by receptor-activated PLCs will directly enhance NMDA receptor-channel activity by phosphorylating the R1 subunit's $Ca^{2+}$·CaM binding site as well as preventing $Ca^{2+}$·CaM from binding to this site and inactivating the receptor. The PKCs will prevent the $Ca^{2+}$ entering the cell from being expelled by blocking the activation of the $Ca^{2+}$ pump by phosphorylating the $Ca^{2+}$·CaM binding/activation site. They will also prevent the neurogranins from re-binding CaM by phosphorylating the CaM binding site. As symbolized by the heavy lettering, the result of the conflicting convergence of CaM, $Ca^{2+}$·CaM, and the mGluR-activated PKCs is to increase and prolong the buildup of $Ca^{2+}$·CaM and thus the activity of LTP-establishing $Ca^{2+}$·CaM-dependent enzymes CaMKIV and CaMAC. Except for CaMKIV ($Ca^{2+}$·CaM-dependent protein kinase IV), the symbols are the same as in Fig. 5.2.



and the postsynaptic membrane must be depolarized to unplug the channel. During low-frequency synaptic transmission, the released glutamate binds to both NMDA and AMPA receptors, but it is the activated AMPA receptors that generate the postsynaptic response by opening their gates for $Na^+$, which depolarizes the membrane.[105–107] The NMDA receptors don't contribute much to the response under these conditions because of their $Mg^{2+}$ plugs.[105–107] However, during high-frequency synaptic transmission the depolarization of the postsynaptic membrane by $Na^+$ flowing through the AMPA receptor channels is great enough to cause the NMDA receptors to spit out their $Mg^{2+}$ plugs and allow $Ca^{2+}$ to flow into the cell to drive LTP.[105–109]

Thus, the AMPA receptor is an important member of the LTP-inducing team. A number of studies have indicated increased AMPA channel function, due to modification of the receptor or to an increase in the number of the receptors at the postsynaptic density during LTP.[110–112] It is believed that while NMDA receptor activation is involved in the induction of LTP, AMPA receptor activation is also required for its expression.[111] Although the AMPA receptor channels are generally not permeable to $Ca^{2+}$, under certain conditions their subunits can be altered to make them permeable to $Ca^{2+}$ and thus able to induce LTP.[111–114] In fact, certain types of neuron express a kind of AMPA receptor that is highly permeable to $Ca^{2+}$, the entry of $Ca^{2+}$ through which can induce LTP.[115] However, the relationship between AMPA receptor-induced LTP (AMPAR-LTP) and NMDA receptor-induced LTP (NMDAR-LTP) is not yet defined.

While NMDA receptor-mediated $Ca^{2+}$ entry is needed to induce most forms of LTP in many types of neurons, $Ca^{2+}$ flowing in through VSCCs also induces some form of postsynaptic plasticity.[24] This has been suggested by the results of experiments in which the activation of VSCCs, while NMDA receptor channels were blocked, was enough to induce LTP in hippocampal CA1 and CA3 neurons.[26,116–118] This LTP is generally termed VSCC-LTP. In many cases, VSCC-induced LTP in CA1 pyramidal and other neurons has been blocked specifically by L-type channel blockers, indicating that $Ca^{2+}$ influx through this kind of channel can trigger LTP induction.[119,120] Activation of L-type channels appears to be necessary for generating $Ca^{2+}$ spikes,[121] and such spikes are believed to be needed for VSCC-LTP.[120–123] It has been suggested that these L-type channels may not by themselves be a good enough $Ca^{2+}$ source for inducing VSCC-LTP, but rather a source of depolarization to activate other VSCCs.[24] Besides L-type channels, other VSCCs, P/Q-, R-type, and low-voltage-sensitive T-type channels have also been implicated in LTP induction.[24]

Yet, another source of $Ca^{2+}$ for triggering synaptic plasticity is the internal stores (Fig. 5.2). Several studies have indicated that presynaptic activity could raise $[Ca^{2+}]_i$ in postsynaptic neurons by triggering the release of $Ca^{2+}$ from internal stores[23] and that this $Ca^{2+}$ could stimulate gene expression and induce synaptic plasticity.[23] Two major mechanisms, $InsP_3$ (inositol-1,4,5- trisphosphate)-mediated $Ca^{2+}$ release and CaCR ($Ca^{2+}$- induced $Ca^{2+}$ release), are believed to be involved in the emptying of the internal $Ca^{2+}$ stores.[23] Activation of postsynaptic G-protein-linked, type 1 metabotropic receptors (mGluR1s) by glutamate can activate a specific phospholipase-C (PLC) that releases $InsP_3$ and diacylglycerol from membrane phosphoinositides.[124] $InsP_3$, acting on its receptors on endoplasmic reticulum, mobilizes intracellular $Ca^{2+}$, and diacyglycerol can activate PKCs, which, as we will see below, are also implicated in LTP induction (Figs. 5.2 and 5.3).[23,124–126] This internally stored $Ca^{2+}$ can also be mobilized

directly by CaCR either by $Ca^{2+}$ interacting with $InsP_3$ receptors or RyRs (ryanodine-sensitive receptors).[23,124] Several studies have indicated that $Ca^{2+}$ spikes not only replenish the internal $Ca^{2+}$ stores, but can also enhance $InsP_3$-induced release of $Ca^{2+}$ from the endoplasmic reticulum.[23,127,128] This coincident increase could occur if postsynaptic depolarization is paired with the synaptic activation of mGluR1 to generate $InsP_3$. Indeed, it has been shown with hippocampal CA1 pyramidal neurons that repetitive mGluR1 receptor activation along with back-propagating action potentials synergistically cause release of $Ca^{2+}$ from $InsP_3$-sensitive stores (Figs. 5.2 and 5.3),[128] and the increase in $[Ca^{2+}]_i$ is a very large (multi-micromolar) surge in apical dendrites. It is suggested that a small increase in the $InsP_3$ level due to metabotropic receptor activation (or for that matter other G-protein coupled receptors linked to PLC) could prime or sensitize the $InsP_3$ receptors on the endoplasmic reticulum to respond to subthreshold $Ca^{2+}$ signals from VSCCs or other receptor-operated channels.[23] A similar sensitization could be obtained by activating the metabotropic receptor-like CaRs by $Ca^{2+}$ dumped into the synaptic space along with the glutamate.[8a,129] It has been shown that parallel fiber inputs into Purkinje cells evoke a localized increase in postsynaptic $[Ca^{2+}]_i$, which is in part due to AMPA receptor-induced depolarization and the other due to mGlu→$InsP_3$-dependent release of $Ca^{2+}$ from endoplasmic reticulum.[130] Such metabotropic receptor-mediated synaptic plasticity has been described in hippocampus,[131,132] cerebellum,[133,134] and visual cortex.[135] mGluR-induced increase in $[Ca^{2+}]_i$ has also been implicated in LTD induction.

As mentioned above, one of explanations of how a rise in $[Ca^{2+}]_i$ can induce the diametrically opposite LTP and LTD is based on the different $Ca^{2+}$-sensitivities of these processes. More $Ca^{2+}$ is needed to induce LTP than LTD, which agrees with the observation that strong high-frequency stimulation induces LTP whereas weak, prolonged low-frequency stimulation induces LTD.[24,84–86] Using potent caged $Ca^{2+}$-chelators, which can be released to modulate $[Ca^{2+}]_i$ surges, it has been shown that a seconds-long postsynaptic elevation of $[Ca^{2+}]_i$ to high levels induces LTP whereas a modest, minute(s)-long elevation induces LTD, suggesting that the amplitude and duration of the $Ca^{2+}$ signal determines the kind of synaptic modification.[136] This is also reflected in the fact that the several $Ca^{2+}$-dependent enzymes implicated in these processes (see below) also have differential $Ca^{2+}$ sensitivity. For example, $Ca^{2+}$/CaM-dependent kinase II (CaMKII), which is believed to be involved in LTP formation, has a lower affinity for $Ca^{2+}$/CaM compared to protein phosphatase 1 that is believed to be involved in LTD formation.[84,137]

What is the molecular basis of $Ca^{2+}$-induced long-term synaptic plasticity? Initial clues from early studies suggested a role for protein kinases in this process. Thus, LTP-inducing stimuli in hippocampus slices enhanced the phosphorylation of specific proteins, while there was no such enhancement by a stimulation that did not induce LTP.[138] This was soon supported by the observation that induction of LTP could be blocked by protein kinase inhibitors.[139,140] It is now clear that a number of $Ca^{2+}$-dependent protein kinases (CaMKII, CaMKIV, MAPK, PKA, PKC, and Src kinases) and phosphatases (calcineurin, PP1) are activated or suppressed during the induction of LTP/LTD and are believed to participate in this process.[104,106,141–145] Understanding how these signaling cascades are interlinked and how the signals they generate collaborate to enhance synaptic transmission has been the major challenge.

NMDA receptor channels, which are the critical sources of $Ca^{2+}$ for LTP induction, are concentrated in the postsynaptic density where they interact with a number of signaling molecules, including $Ca^{2+}$, CaM, and several protein kinases, through cytoskeletal scaffolding consisting of actin and associated proteins such as postsynaptic density-95 (PSD95) and spinophilin.[106] Similarly, AMPA receptors and VSCCs also interact, either directly or through the cytoskeletal scaffolding, with various signaling molecules which modulate channel activity and link the surface $Ca^{2+}$ signal to protein syntheses and gene expressions which appear to be needed for some forms of long-term synaptic plasticity.

One of the protein kinases that is strongly implicated in enhanced postsynaptic transmission is CaMKII,[106,146] which is found in high concentrations in post synaptic densities (Figs. 5.2 and 5.3).[147–148] This is an oligomeric enzyme that is activated upon binding of $Ca^{2+}$·CaM,[147] which causes the enzyme to autophosphorylate into a constitutively active, $Ca^{2+}$·CaM-independent form.[147] The active autophosphorylated enzyme then associates with postsynaptic densities, at least partly by binding to NMDA receptors.[149–151] Thus, CaMKII activity stays high long after the $Ca^{2+}$ signal has passed, which fits with $Ca^{2+}$ triggering rather than maintaining long-term synaptic plasticity. It has been shown in cultured hippocampal and other neurons that increasing $[Ca^{2+}]_i$ by activating NMDA receptors causes CaMKII to constitutively activate itself by autophosphorylating its $Thr^{286}$.[106,150,152] Indeed, CaMKII inhibitors or CaM antagonists block LTP-induction in CA1 pyramidal neurons.[139,153–155a] Furthermore, the $Thr^{286}$→Ala mutation, which eliminates CaMKII's ability to phosphorylate itself, inhibits NMDAR-induced LTP in CA1 and other hippocampal neurons.[156]

How could CaMKII activity affect postsynaptic plasticity? Among CaMKII's targets are the AMPA receptors that are often found alongside NMDA receptors in the postsynaptic densities.[100,147,148] The phosphorylation of the GluR1 subunit of AMPA receptors by CaMKII has been seen in cultured hippocampal neurons[157] and CA1 pyramidal neurons,[114] and it is known to increase the ion conductance of the receptors' channels.[106,158,159] Activating NMDA receptors has been shown to stimulate the phosphorylation of the AMPA receptors' GluR1 subunit at its specific CaMKII phosphorylation site in cultured hippocampal cells.[157] The same phosphorylation has been observed following the induction of LTP in area CA1,[114] suggesting a critical role for CaMKII-induced phosphorylation of AMPA receptor in LTP induction. The importance of the GluR1 subunit and its phosphorylation in LTP has been confirmed by the absence of LTP in the CA1 pyramidal cells of transgenic mice without GluR1 subunits in their AMPA receptors.[111]

It is suggested that with low-frequency stimulation, there is only a modest increase in $[Ca^{2+}]_i$, which is believed to induce LTD by generating just enough $Ca^{2+}$·CaM complexes to activate the protein phosphatase, calcineurin, but not enough to activate CaMKII.[84,137] Calcineurin in turn activates protein phosphatase 1 (PP1) by dephosphorylating and thus inactivating the PP1-inhibiting I-1.[137] PP1 could then inhibit LTD induction by switching off CaMKII by dephosphorylating the kinase's phospho-$Thr^{286}$. So how does the larger $Ca^{2+}$ influx needed to activate CaMKII activity and drive LTP suppress PP1 activity? The answer is cyclic AMP: it plays a critical part in determining the nature of long-term synaptic plasticity. Cyclic AMP swings into action when the $Ca^{2+}$ influx through NMDA receptors activates the type 1 adenylyl cyclase that makes it. Its job is to activate the protein kinase, PKA, which we have met often in other parts of

our tour.[160] PKA then phosphorylates I-1, which then binds to, and inhibits, PP1.[161] This promotes LTP by preventing the dephosphorylation and inactivation of autophosphorylated CaMKII.[161] This link between PKA, PP1, and LTP has been suggested by experiments in which PKA inhibitors were found to block LTP and that this block was lifted by inhibiting PP1 or calcineurin.[161–164] Thus, there is a delicately poised balance between protein kinase and phosphatase activities, the tipping of which in one or the other direction can determine what kind of synaptic plasticity is induced.

Of course, this is not all! Members of another family of kinases — the conventional $Ca^{2+}$-dependent PKCs — are also very important players in LTP.[106,165–167] The results from several studies have indicated that these PKCs are persistently activated and phosphorylate AMPA and NMDA receptors during LTP induction.[165] The NMDA receptor's R1 (NR1) subunit, like several other proteins,[168] has a $Ca^{2+}$·CaM binding domain that is also a PKC target site (Fig. 5.3).[169,170] $Ca^{2+}$·CaM binding and phosphorylation of this shared target domain by PKCs are mutually exclusive — binding of $Ca^{2+}$·CaM to this domain prevents PKC from phosphorylating it while PKC phosphorylation prevents $Ca^{2+}$·CaM from binding to it.[168–170] Phosphorylation of the R1 subunit by PKCs increases the probability of the NMDA receptor's channel opening and thus the influx of $Ca^{2+}$ and the generation of $Ca^{2+}$·CaM by promoting the ejection of the $Mg^{2+}$ plug.[168–170] The persistently activated PKCs associated with LTP induction would keep the $Ca^{2+}$ flowing through the channels by preventing the $Ca^{2+}$·CaM it makes from feeding back and closing the NMDA channels. The PKCs do this by phosphorylating the $Ca^{2+}$·CaM binding site on the NMDA receptors' NR1 subunits and preventing $Ca^{2+}$·CaM from binding to the sites and closing the channels.[168] The PKCs would also home in on and phosphorylate the $Ca^{2+}$-ejecting pump's inhibitory domain and prevent $Ca^{2+}$·CaM from binding onto it.[171–174] This would further increase the size of the $Ca^{2+}$ surge by preventing the $Ca^{2+}$·CaM complexes from lifting the block and turning on the pump to expel the excess $Ca^{2+}$.[168] Another PKC substrate that may be involved in LTP is neurogranin, a postsynaptic protein which binds CaM instead of $Ca^{2+}$·CaM in the PKC target domain.[175,176] Neurogranin promptly releases its CaM as $Ca^{2+}$·CaM when $Ca^{2+}$ comes surging in through the NMDA receptors or VSCCs (Figs. 5.2 and 5.3).[175] A persistently enhanced phosphorylation of neurogranin has been seen in hippocampal neurons during LTP, which correlates to the persistent PKCs activation.[177,178] Presumably, the $Ca^{2+}$ surging through the glutamate-activated NMDA receptors in these cells releases CaM from neurogranin and the resulting $Ca^{2+}$·CaM spreads out to stimulate key players in LTP induction such as CaMKII, CaMKIV, and adenylyl cyclase (Fig. 5.3).[168] By homing in on and phosphorylating neurogranin's CaM binding domain, the persistently active PKCs would keep the level of free CaM up to feed $Ca^{2+}$·CaM formation by the $Ca^{2+}$ flowing through the NMDA channels which the kinases have locked open. Clearly, the conflicting convergence of PKCs, CaM, and $Ca^{2+}$·CaM on their shared domains in key enzymes, pumps, sequestering proteins, and receptor channels has a very powerful, although as yet inadequately appreciated, impact on the response of various mechanisms, including gene transcription to the $Ca^{2+}$ signal surges that drive LTP formation.[168]

Beside adenylyl cyclase, CaMKII, PKCs, and PKA, tyrosine kinases and MAP kinases have also been implicated in postsynaptic plasticity.[106,142,165] The NR2A and 2B subunits of NMDA receptors are known to be phosphorylated by tyrosine kinases.[142,179,180] This phosphorylation is believed to relieve the $Zn^{2+}$-inhibition of the receptors and

increase the current flowing through them.[181] Elevated tyrosine phosphorylation of NMDA receptors by Src kinase has been observed during LTP.[182,183] Indeed, selectively inhibiting Src kinase blocks LTP.[184,185] Src kinase is certainly strategically placed to affect LTP. It is located in the postsynaptic densities, and it must somehow be tethered to NMDA receptors because it can be immunoprecipitated along with them by anti-NMDA receptor antibody.[186] Another Src-like kinase implicated in NMDA receptor phosphorylation is Fyn tyrosine kinase.[186–188] It is generally believed that the enhancement of NMDA receptor conductance by tyrosine phosphorylation increases $Ca^{2+}$ influx into the postsynaptic dendrites, which in turn potentiates the AMPA-receptor-mediated current leading to enhanced synaptic activity.[184] There is evidence that activation of AMPA receptors can induce MAP kinase activity, which may play a role in LTP.[189] It is conceivable that $Ca^{2+}$-dependent activation of this motley band of kinases ultimately homes in on one target, the CREB (cAMP-response element binding protein) family of transcription factors, which stimulate the expressions of specific genes, the products of which are required for long-term synapse modification.[190–192]

## Calcium and neurotransmitter release

In the preceding section, we saw how $Ca^{2+}$ regulates synaptic plasticity. Now we will see how a rise in $[Ca^{2+}]_i$ can modulate the presynaptic release of transmitters that trigger synaptic plasticity. A large rise in the presynaptic $[Ca^{2+}]_i$ from a basal 0.1 mM to as much as 200 μM[24,40,41,193–195] is a major part of the complex mechanism that regulates neurotransmitter release. As we have just learned, the major presynaptic ports of entry of $Ca^{2+}$ are the VSCCs. Both N- and P/Q-type $Ca^{2+}$ channels are involved in triggering neurotransmitter release from synaptic vesicles in both the central and peripheral nervous systems.[35–36a,42,196,197] The finding that these channels interact with neurotransmitter-loaded synaptic vesicles with their α-subunits in nerve terminals as opposed to dendritic spines strongly supports this involvement.[42,198–202] $Ca^{2+}$, surging through these channels when they are opened by the arrival of the action potential at the terminal, boots up a complex mechanism that pulls the synaptic vesicles down onto the presynaptic membranes, initiates their fusion with the membrane and finally dumps their load of transmitters into the synaptic cleft.[199–204]

In most eukaryotic cells, molecular products addressed for export are packaged into membrane vesicles which fuse with the plasma membrane and dump their contents into the extracellular milieu — a process known as exocytosis.[203,204] Regulated exocytosis is the basis of neurotransmission which includes the release of neurotransmitters and neuromodulators.[205–209] In neurons, these transmitters and modulators are packaged in at least two different organelles — small synaptic vesicles (SSVs), which generally contain the so called 'fast' neurotransmitters (e.g., glutamate, GABA, glycine, aspartate, serotonin, and acetylcholine) and large dense-core vesicles (LDCVs), which contain the 'slow' transmitters (neuropeptides).[203] The contents of these vesicles are released by $Ca^{2+}$-driven exocytosis, with the SSVs needing a lot of $Ca^{2+}$ and the LDCVs requiring much less to be dumped.[203] In neurons, most of the synaptic vesicles, the 'reserve pool,' are held back from the synaptic membrane in a filamentous cage and only a small proportion, the RRP ('readily releasable pool'), is kept near the release sites where they are very near or actually attached to the terminal's $Ca^{2+}$ channels where the $Ca^{2+}$ hot spots can form

when the ions start flowing through the channels.[205,210] It is these frontline vesicles that promptly release their loads of transmitter (e.g., amino acids and acetylcholine) upon the arrival of the channel-opening action potential. Neurotransmitter dumping is the work of a highly specialized signal-booted membrane trafficking cycle, which includes vesicle formation, recruitment, targeting to release sites, docking, fusion, and recycling, many parts of which it shares with constitutive membrane trafficking.[205,210]

First, we must look at $Ca^{2+}$'s roles in vesicle formation and recruitment to the RRP. $Ca^{2+}$ seems not to be involved in vesicle formation, but it is involved in a big way in vesicle recruitment. In order to avoid RRP depletion at the terminal, synaptic vesicles in the 'reserve pool' must be released from their cage and sent to the RRP. How is this done? As we learned above, synaptic vesicles are tethered to cytoplasmic microfilaments (e.g., actin). Although the details of this tethering are unknown, a vesicle-associated protein, synapsin, is somehow involved in it.[211–214] There are five synapsin isoforms (Ia, Ib, IIa, IIb, and IIIa), many of which are substrates of $Ca^{2+}$-regulated protein kinases.[210,212,215–217] Phosphorylation and dephosphorylation are believed to control the release of synaptic vesicles from the reserve pool and their transfer to the RRP.[210,212] A number of kinases, CaMKI and II, PKA, PKCs, and MAPK are known to phosphorylate the N- and C-termini of the synapsins.[212,214,218–220] Dephosphorylated synapsin I associates with the cytoskeleton and ties the reserve-pool synaptic vesicles to the filamentous cage.[210,212,221] $Ca^{2+}$ causes synapsin I to change its shape, reduce its affinity for synaptic vesicles as well as cytoskeletal actin filaments. This reconfiguration releases the vesicle from the filaments.[210,222] But how might $Ca^{2+}$ do this? $Ca^{2+}$ surging through the terminal channels upon the arrival of an action potential makes $Ca^{2+}$-CaM, which activates CaMKII, which is also attached to the synaptic vesicles. CaMKII liberates the vesicles from the reserve pool by phosphorylating synapsin's C-terminus.[210,223] Several studies support this mechanism. For example, presynaptic injection of CaMKII into a squid's giant axon synapse enhances neurotransmitter release.[224] Furthermore, injecting unphosphorylated synapsin I inhibited transmitter release, which was reversed by phosphorylating synapsin with CaMKII, but not with CaMKI or PKA.[224] Although CaMKII-mediated phosphorylation may play the biggest role in the release of vesicles from the reserve pool, recent studies have suggested that synapsins may also be phosphorylated by other kinases. A role for PKA and CaMKI in the phosphorylation of synapsin is suggested by the ability of the phosphorylation of the protein's N-terminal PKA and CaMKI phosphorylation sites to release synaptic vesicles. Indeed, a $Ca^{2+}$ surge into the terminal can activate $Ca^{2+}$-dependent adenylate cyclase and thus PKA as well as CaMKI, both of which could release vesicles from the 'reserve pool'.[212] Then, of course, there are the PKCs. Activation of the $Ca^{2+}$-dependent conventional PKCs may increase both the size and rate of RRP replenishment in hippocampal cultures.[225] Some studies indicate that synapsins are also PKC substrates, but it is not certain whether this PKC-stimulated RRP replenishment is mediated through synapsin phosphorylation. Indeed, it is likely that there may be a parallel mechanism.

After their release from the 'reserve pool' by $Ca^{2+}$-stimulated phosphorylation, the synaptic vesicles have to be targeted, docked, and primed to join the RRP at the active sites on the presynaptic membranes. Nature is notoriously conservative, so neurons use the same, but much speeded up, mechanism that drives specific membrane fusion and exocytosis in other eukaryotic cells. A key part of the fusion machinery is the so-called

20S-particle or complex, a product of the specific interactions of the proteins on the vesicles and the target membranes. It includes NSFs (*N*-ethylmaleimide-sensitive fusion proteins)[226–229] and SNAPs (soluble NSF-attachment proteins).[227,228,230] SNAPs bind to receptors on the vesicles and the target membranes that are collectively called SNAREs (soluble NSF-attachment protein receptors).[229–231] Depending on where they are, the SNAREs on synaptic vesicles are called v(vesicle)SNAREs (found on synaptic vesicles) and the SNAREs on the plasma membranes are called t(target)SNAREs.[229,231*a*–233] The original SNARES were VAMP (synaptolbrevin), syntaxin, and SNAP-25.[234–238] According to the SNARE hypothesis,[238*a*–240] the v(vesicle)-SNARE VAMP (synaptobrevin), attached to the vesicle with its C-terminus inserted into the vesicle membrane, binds to the t(terminal)SNARE consisting of the axon terminal membrane-bound (by its C-terminus inserted into the membrane) syntaxin and its associated SNAP-25 to produce a stable 7S "docking" complex. Although syntaxin can by itself associate with VAMP(synaptobrevin), SNAP-25 substantially enhances the association.[241,242] This 7S vSNARE·tSNARE complex provides a high-affinity receptor for SNAPs, which in turn bind NSFs to make the 20S fusion complex.[231,231*a*,243] By itself, NSF is a weak ATPase but its activity rises when it binds SNAPs.[244,245] The SNAP-activated NSF-mediated ATP hydrolysis primes the docked vesicles for $Ca^{2+}$-triggered fusion and transmitter dumping,[244] possibly by causing the disassembly of the VAMP·syntaxin·SNAP complex.[245] Besides being attached to VAMP(synaptobrevin), the SNAP-25·syntaxin complex also interacts with a N-type $Ca^{2+}$ channel's synprint (synaptic protein interaction) site.[246–248] This interaction puts the membrane-tethered synaptic vesicles alongside the $Ca^{2+}$ entry port where the ephemeral high-$Ca^{2+}$ hot spots appear.

How does $Ca^{2+}$ persuade the now 'docked and primed' vesicles to fuse with the plasma membrane and dump their transmitters? Many $Ca^{2+}$-sensor proteins are implicated in this process.[249] Among them are the four (I, II, IV) synaptotagmin isoforms, most of which are highly expressed in neurons.[250,251] These are low-affinity $Ca^{2+}$ binding proteins found associated with synaptic vesicles,[250,251] hence they are grouped with the vSNAREs. They bind to membrane phospholipids and the tSNARE syntaxin in a $Ca^{2+}$-dependent manner.[251–254] Synaptotagmins have two $Ca^{2+}$-binding C2 domains, C2A (at the membrane) and C2B (away from the membrane).[232,254–255*a*] At the resting $Ca^{2+}$ concentration, synaptotagmin tethers are believed to hold the vesicles in the RRP close to the cell membrane by binding to the vesicle's SV2 protein with their C2B domains and to the tSNARE proteins on the cell membrane with their C2A domains.[200,251,256] When $Ca^{2+}$ surges through the adjacent channel, it binds to the C2A domain and sharply shoves the protein deeper into the membrane phospholipid bilayer,[200] as happens with all other proteins carrying C2 domains. On the other vesicle's end, the C2B domain dissociates from SV2,[257,258] and the membrane-insertion of C2A domain induces synaptotagmins to cluster through the $Ca^{2+}$-dependent interaction between their C2B domains.[259–261] This clustering of SNARE-linked synaptotagmins is believed to organize a vSNARE complex at a single locus on the vesicle, which enables better fusion of the vesicle with the membrane.[200] The $Ca^{2+}$-driven plunge of the C2A domain into the membrane is believed to facilitate rapid fusion of synaptic vesicles in a number of ways. It pulls vesicles to the synaptic membrane by facilitating the "zippering" together of the vesicle-bound VAMP(synaptobrevin) and the cell membrane's tSNARE (syntaxin·SNAP-25) complex.[207,238*a*] The vesicle is pulled down to the axon terminal membrane by the

simultaneous shortening of the C-terminal regions of syntaxin and synaptobrevin.[238a] Furthermore, the insertion of the C2A domain into the membrane may promote vesicle fusion either by perturbing the membrane lipid bilayer and altering the physical relationship between SNAREs and membrane lipids or by inducing conformational/structural changes in SNAREs.[200] Although the precise mechanism of $Ca^{2+}$-induced vesicle fusion is not clear, recent kinetic studies have clearly established that synaptotagmin C2 domains are designed to detect and respond rapidly to both rises and falls in the $Ca^{2+}$ levels at the synaptic terminals and thus are ideally suited to regulate synaptic vesicle exocytosis and endocytosis.

## $Ca^{2+}$ and neuronal cell death

While $Ca^{2+}$ is essential for neuroblast proliferation, driving axon growth cones, and neurotransmitter secretion, it can also kill neurons just as we saw it killing colon cells and keratinocytes during the normal course of diffpoptosis. However, the neurons left after the extensive culling in the developing brain are meant to live a long time, which requires a careful control of the intracellular $Ca^{2+}$ level.[262–264] Since the early observation that amputated axons must have extracellular $Ca^{2+}$ to die,[265] much evidence of the ion playing the lead role in the killing of neurons under various conditions has been collected. Indeed, the glutamate-driven excitotoxicity, oxidative stress, mitochondrial dysfunction, and apoptosis that have been proposed to kill neurons in stroked brains, and the degenerating brains of Alzheimer, Huntington, and Parkinson patients are all $Ca^{2+}$-dependent.[266–277] This $Ca^{2+}$-dependence of neuronal killing has given rise to the '$Ca^{2+}$ hypothesis' of neurodegeneration,[267,278] which simply means that $Ca^{2+}$ overload kills neurons. Although there are some exceptions,[279–282] the "$Ca^{2+}$ hypothesis" appears to apply to most, if not all, kinds of neurodegeneration. So how are neurons overloaded with $Ca^{2+}$ and how does it kill them?

Normally, the $[Ca^{2+}]_i$ is held around 100 nM in resting neurons.[23,263,283] As we have just seen, local rises in $[Ca^{2+}]_i$ during neuronal stimulation drive neurotransmitter release and synaptic plasticity. An increase in the $[Ca^{2+}]_i$, which can reach μM levels,[30,40,41] is usually only transient because of prompt binding to intracellular $Ca^{2+}$-buffers, pumping into storage vesicles and mitochondria, and expulsion from the cell by $Ca^{2+}$-pumps or transporters in the plasma membrane.[34a,262,284] Thus, internal $Ca^{2+}$ homeostasis is achieved by strictly balancing $Ca^{2+}$ influx with expulsion and internal sequestration. It is believed that a serious disturbance of this balance leads to $Ca^{2+}$-overload, which is the prime suspect in the murder of neurons in many neurodegenerative disorders. Although how $Ca^{2+}$ overload kills neurons is far from being defined, we know that it triggers the abnormal activity of a host of $Ca^{2+}$-dependent enzymes and cellular processes. It is believed that a multipronged attack by surging $Ca^{2+}$ on many signaling enzymes, including endonucleases, nitric oxide synthase, protein kinases, protein phosphatases, phospholipases, and proteases, generates lethal ROS (reactive oxygen species) and switches on the "death genes."

As we learned in the preceding section, the major sources of $Ca^{2+}$ in neurons are membrane $Ca^{2+}$-channels, such as VSCCs and LGCCs, and the endoplasmic reticulum stores.[23,262,285,286] Neurons, like other cells, have many ways of ensuring that the $Ca^{2+}$ signal lasts no longer than needed to trigger the physiological response. As we have seen

earlier, $Ca^{2+}$ influx through the channels creates $Ca^{2+}$ hot spots which quickly vanish as the ions diffuse away.[24,34,43,287] This rapid disappearance of the hot spots is the work of $Ca^{2+}$ binding proteins such as calbindin, calmodulin, and parvalbumins, as well as many cytoskeletal proteins.[43,288–291a] These buffering proteins have two important jobs — to put the $Ca^{2+}$ surges where they are needed and then to keep them brief.[23,288–292] It is not clear whether a defective functioning of these buffers contributes to the $Ca^{2+}$-overloading of neurons. Some studies have indicated that $Ca^{2+}$ binding proteins such as calbindin actually protect neurons from being killed by excessive excitation (excitotoxicity).[293] Other studies have found that neurons expressing $Ca^{2+}$ buffers such as parvalbumin or calbindin are more susceptible to injury than neurons without them.[294,295]

$Ca^{2+}$ binding proteins have only limited buffering capacities and cannot reduce a $Ca^{2+}$ surge below a certain level. Therefore, to reduce a large $Ca^{2+}$ surge, neurons must rely on their endoplasmic reticula and mitochondria, which can hold large amounts of $Ca^{2+}$.[34a,263,284,296–298] These organelles have two jobs in a neuron — buffering $Ca^{2+}$ by sequestering it and releasing it when needed for various functions.[284,296] $Ca^{2+}$ is pumped into the endoplasmic reticulum by a $Ca^{2+}/Mg^{2+}$-dependent ATPase known as SERCA (sarco/endoplasmic reticulum $Ca^{2+}$ ATPase).[23,263,284,299] There are many isoforms of this enzyme, two (SERCA2b and SERCA3) of which are highly expressed in brain.[300] After SERCA has pumped it into the endoplasmic reticulum, the $Ca^{2+}$ is kept there by binding to calbindin and calreticulin.[291,301] In addition to SERCA, the endoplasmic reticulum has the channels of $InsP_3$ receptors through which the stored $Ca^{2+}$ can be released when the neuron needs it to respond to stimulants.[23,284] As we have seen in the preceding section, $Ca^{2+}$ escapes from the endoplasmic reticulum through these channels when they are opened by $InsP_3$ from the hydrolysis of membrane phosphoinositides by the phospholipase-C stimulated by glutamate-activated metabotropic receptors.[124] The $Ca^{2+}$ released from the endoplasmic reticulum (or for that matter flowing in through plasma membrane $Ca^{2+}$ channels) can trigger a further release of stored $Ca^{2+}$ (the so-called CICR — $Ca^{2+}$-induced $Ca^{2+}$ release) by activating RyRs (ryanodine receptors).[23,284]

A cytoplasmic $Ca^{2+}$ surge above a certain level is sensed by the SERCAs, which pump the excess $Ca^{2+}$ back into the endoplasmic reticulum.[299] It is not clear how the SERCAs detect $Ca^{2+}$ surges in neurons, but CaMKII is likely to be the sensor. Heart muscle SERCA is turned on either when it is phosphorylated or when its suppressor, the protein phospholamban, is inactivated by phosphorylation.[302–305] The brain's SERCA2b is likely activated when phosphorylated by CaMKII, of which neurons have a lot.[146–152] Thus, it seems likely, although it remains to be proven, that when the $[Ca^{2+}]_i$ surges upwards the CaMKII surge sensor turns on SERCA2b, which pumps the excess $Ca^{2+}$ into the endoplasmic reticulum where it binds to calbindin and calreticulin.[299]

The value of the SERCA pumps for regulating neuronal $[Ca^{2+}]_i$ has been demonstrated by the fact that switching them off with thapsigargin prolongs and increases $Ca^{2+}$-induced depolarization.[284,306,307] Moreover, thapsigargin can empty a neuron's endoplasmic reticulum $Ca^{2+}$ stores and trigger apoptosis.[308–311] However, it is not known whether it is the emptying of the endoplasmic reticulum $Ca^{2+}$ stores or the rise in $[Ca^{2+}]_i$ that follows the silencing of the SERCAs that damages the neuron. There is some evidence to suggest that it is the depletion of endoplasmic reticulum $Ca^{2+}$ that is toxic because dantrolene, an inhibitor of ryanodine receptor-triggered release of endoplasmic

reticulum $Ca^{2+}$, can prevent thapsigargin-induced apoptosis in neurons.[309] In fact, dantrolene has been shown to reduce ischemia-induced damage in neurons under several experimental conditions.[312,313] Furthermore, stroke-induced ATP shortage empties the endoplasmic reticulum $Ca^{2+}$ by shutting down the ATP-fueled SERCAs. Such a depletion of the neuron's endoplasmic reticulum $Ca^{2+}$ stores inhibits protein synthesis,[298] which can produce a severely damaging stress response like that caused by stroke.[298] According to the results of some studies, parts of the brain that are especially vulnerable to damage (such as the CA1 region of the hippocampus) have many $InsP_3$ receptor channels and few SERCAs.[298] Consequently, an $InsP_3$-mediated release of $Ca^{2+}$ following ischemia would efficiently and prolongedly empty the $Ca^{2+}$ stores because of a lack of SERCAs to refill them.[298] In support of this, the results from some studies have indicated that activators of the $InsP_3$-generating mGluR1/5 metabotropic glutamate receptors kill neurons, while their inactivation them protects them.[314–316] Moreover, injecting a mGluR1 agonist into the cerebral ventricles triggers seizures and kills neurons.[317] Thus, regardless of whether it be the depletion of the $Ca^{2+}$ stores per se or the resulting increase in $[Ca^{2+}]_i$ that is important, endoplasmic reticulum $Ca^{2+}$ appears to play an important role in the killing of neurons.

The neuron's mitochondria can also hold a lot of $Ca^{2+}$. It is becoming increasingly evident that mitochondrial uptake and release of $Ca^{2+}$ plays an important role in neuron function.[284,297,318,319] We will see below that mitochondrial $Ca^{2+}$, $Ca^{2+}{}_m$, plays a big part in the killing of neurons in several neurodegenerative disorders. Mitochondria, like the endoplasmic reticulum, can sequester $Ca^{2+}$, although their affinity for $Ca^{2+}$ is lower and they require a higher $[Ca^{2+}]_i$ to induce them to load up with the ion.[297,318,320–322] The inner mitochondrial membrane is involved in the selective uptake and release of ions through specific channels and transporters. $Ca^{2+}$ is brought into the mitochondrion by a specific low-affinity, high-capacity $Ca^{2+}$ uniporter whose activity is highly dependent on the extramitochondrial $Ca^{2+}$ concentration. Thus, when the cytoplasmic $[Ca^{2+}]$ rises above a threshold or set-point level , the uniporter can carry much of this $Ca^{2+}$ into the mitochondria.[297–323] This $Ca^{2+}$ uptake is largely driven by the mitochondrial membrane potential, $\Delta\psi$.[297,318,320–322] Calcium is expelled from a mitochondrion mainly by the $Na^+/Ca^{2+}$ exchanger.[319,324] The activity of $Na^+/Ca^{2+}$ exchanger is in turn coupled to the $Na^+/H^+$ exchanger that extrudes $Na^+$ from the mitochondrion at the expense of the respiration-generated pH gradient, $\Delta pH$.[297] Thus, the flow of protons into the mitochondrial matrix to make ATP creates a counterflow of $Na^+$ out of the matrix via the $Na^+/H^+$ exchanger, which in turn creates a counterflow of $Ca^{2+}$ out of the matrix as the ejected $Na^+$ flows back into the matrix via the $Na^+/Ca^{2+}$ exchanger. The pH gradient across the inner mitochondrial membrane is maintained by respiration and drives ATP production by the ATP synthase. The $\Delta pH$ and the $\Delta\psi$ together constitute the mitochondrial proton electrochemical gradient, $\Delta p$.[319,323,325,326] ATP synthesis requires a flow of positively charged protons (that have been pumped into the intermembrane space by the four respiratory complexes to create the $\Delta pH$) through the rapidly spinning ADP-Pi-linking F1 heads of the ATP synthase generators into the more negative matrix.[326a,b] The proton flow (i.e., its 'amperage') through the generators is a function of the transmembrane proton (pH) gradient and the overall transmembrane charge gradient (potential) $\Delta\psi$. Should the proton flow stop, the synthase changes into an ATP-eating ATPase. Obviously, $\Delta\psi$ determines mitochondrial $Ca^{2+}$ uptake and release. As we shall see below, factors affecting

$\Delta\psi$ and mitochondrial energy metabolism can cause mitochondrial $Ca^{2+}$ overload, which triggers a series of biochemical events that kill the neuron.

The plasma membrane has the major ports of $Ca^{2+}$ entry, such as the VSCCs and LGCCs, as well as the machinery to expel $Ca^{2+}$ from the cytosol to keep the neuron's $[Ca^{2+}]_i$ within safe limits. There are two major $Ca^{2+}$-expelling devices — the ATP-fueled $Ca^{2+}$ pump and the $Na/Ca^{2+}$ exchanger, which depends on the normally steep inwardly directed $Na^+$ gradient to drive a counterflow of $Ca^{2+}$ out of the cell.[327–329] The plasma membrane $Ca^{2+}$ pump is a $Ca^{2+}$ ATPase[328,329] like the endoplasmic reticulum's SERCAs, but it differs from the SERCAs in being activated by $Ca^{2+}$·CaM.[328,329] The plasma membrane pump uses one ATP to eject one $Ca^{2+}$, while a SERCA uses one ATP to pump two $Ca^{2+}$ ions into the endoplasmic reticulum.[262] The activity of the plasma membrane pump is also regulated by phosphorylation by protein kinases such as PKA and PKC, as well as a number of fatty acids.[262,330,331] $Ca^{2+}$ flowing through opened membrane channels or released from internal stores produces $Ca^{2+}$·CaM complexes that switch on (or more accurately de-inhibit) the $Ca^{2+}$ pumps.[168] The excess cytosolic $Ca^{2+}$ is pumped out and the resting $Ca^{2+}$ level is restored. The $Na^+/Ca^{2+}$ exchanger also senses a rise in $[Ca^{2+}]_i$ and exploits the inwardly directed $Na^+$ gradient to expel one $Ca^{2+}$ ion for every 2–3 $Na^+$ flowing into the cell.[266,327] The incoming $Na^+$ is then detected and expelled by the $Na^+/K^+$ ATPase pumps if and when it reaches a critical level.[262,327] Since ATP fuels both of the neuron's $Ca^{2+}$-expelling devices, a reduction of ATP production can produce a lethal $Ca^{2+}$-overload.

The neuron killer in many neurodegenerative diseases is excitotoxicity — the lethality of too much stimulation by a neurotransmitter such as glutamate.[262,267,268] Glutamate is a major neurotransmitter in the central nervous system and is present in millimolar concentrations in the gray matter.[262] The concentration of glutamate in the extracellular space is normally kept at a very low level.[332] However, when neurons are stimulated, glutamate is dumped into the synaptic space in boluses or quanta (quantal release), which bind to various postsynaptic receptors that trigger diverse neuronal responses, most of which, including synaptic plasticity and transmitter release, are $Ca^{2+}$-dependent. The released glutamate must not be allowed to stay around for too long and persistently activate receptors. It is quickly sucked back up into glial cells and neurons by their glutamate transporters that are driven by the same transmembrane $Na^+$ gradient that drives the $Na^+/Ca^{2+}$ exchanger.[332–334] However, under certain conditions, such as the ischemia in a stroked brain, enough glutamate is dumped out of the presynaptic neurons to overwhelm the transporters and stay outside the cell where they overload the postsynaptic neurons with $Ca^{2+}$ by persistently stimulating their various glutamate receptors.[262] It is this persistent glutamate signaling that kills neurons. How does $Ca^{2+}$ overload occur under these conditions and how does it kill neurons?

As we have learned above, glutamate activates its ionotropic NMDA, AMPA, and kainate receptors (so called because they admit $Na^+$, $K^+$, and $Ca^{2+}$ ions into the cell) and opens their ion channels,[335–337] and it activates the G-protein-coupled, $InsP_3$-generating metabotropic receptors that are related to the CaRs we met so often in the previous chapters and, as we have seen, mobilize $Ca^{2+}$ from the endoplasmic reticulum.[23,262] While the $Ca^{2+}$-admitting NMDA receptor channels are the principal instruments of glutamate-induced neurotoxicity,[335] other glutamate receptors, such as the AMPA receptors, become accessories to the crime.[267,338,339] For example, AMPA receptors, which normally

admit only $Na^+$, can admit $Ca^{2+}$ under certain conditions such as the ischemia of stroke where their subunit composition changes.[340–342] Besides the failure to sweep up released glutamate to stop the flow of $Ca^{2+}$ through the NMDA receptor channels, the neuron suffers a from a growing fuel shortage during ischemia.[272,297,343] This energy shortage, which is due to the depletion of the cellular ATP pool, also contributes to the $Ca^{2+}$ overload. The ATP shortage shuts down the plasma membrane's $Na^+/K^+$ ATPase, which causes the $Na^+$ gradient to collapse and thus depolarize the neuron's plasma membrane.[262,327,344] (It should be noted that the $Na^+/K^+$ ATPase is a major fuel consumer that uses about 40% of a neuron's ATP to maintain the ionic gradient and the plasma membrane potential.)[344] The collapse of the $Na^+$ gradient disables the gradient-driven glutamate transporter, the consequence of which is a buildup of extracellular glutamate and its signaling to a lethal level. The depolarization stimulates $Ca^{2+}$ influx in two ways — it opens VSCCs and blows the $Mg^{2+}$ plugs out of NMDA receptors, which enables $Ca^{2+}$ to flow through them when they are activated by the accumulating glutamate.[103–105] The $Ca^{2+}$·CaM made by the rising $[Ca^{2+}]_i$ should turn on the plasma membrane's $Ca^{2+}$ pumps to expel the excess $Ca^{2+}$,[168,328,329] but the cell is running out of ATP fuel to operate them. As if this were not enough, the fuel shortage and the collapse of the $Na^+$ gradient cause the $Na^+/Ca^{2+}$ exchanger to work in reverse and bring more $Ca^{2+}$ into the increasingly stressed neuron! The excessive glutamate also simultaneously activates the G-protein-linked metabotropic glutamate receptors that stimulate protein kinases such as PKCs,[124] which can phosphorylate and keep the plasma membrane $Ca^{2+}$ pumps from working by preventing $Ca^{2+}$·CaM from binding to them and turning them on.[168] The PKCs have another dangerous capability — they can keep the glutamate-activated NMDA receptors open and passing $Ca^{2+}$ by phosphorylating the $Ca^{2+}$·CaM binding site on the receptors' NR1 subunits, which prevents them from being shut down by the $Ca^{2+}$·CaM complexes produced by the incoming $Ca^{2+}$.[168] As we have seen earlier, a rise in $[Ca^{2+}]_i$ can also activate other protein kinases such as PKA, CaMKII, and tyrosine kinases, which are known to activate both VSCCs and LGCCs.[104,106,141–145] Although it is far from clear as to how efficiently protein kinases can phosphorylate and activate ion channels when the ATP level is low, it is obvious that a combination of open $Ca^{2+}$ channels and disabled $Ca^{2+}$ extrusion systems results in $Ca^{2+}$ overloading of neurons.

One of the major consequences of the disruption of $Ca^{2+}$ homeostasis is the induction of oxidative stress by the excessive generation of ROS, the various reactive oxygen species, which kill neurons in many neurodegenerative disorders.[319,322,345–347] ROSs modulate the opening of many ion channels, including $Ca^{2+}$ channels, which allows the entry of more $Ca^{2+}$, which in turn generates more ROS, thus completing a lethal vicious cycle.[267,348,349] Although mitochondria are by far the largest sources of ROS, the cyclooxygenase (COX) pathway, which makes prostaglandins, and the microsomal NADPH cytochrome P450 reductase system also generate them.[350,351] The sustained elevation of $Ca^{2+}$ can overactivate cytosolic phospholipase A2 (cPLA2), which releases arachidonic acid (AA) from membrane phospholipids.[347,350,352] Arachidonic acid can generate ROS during its conversion to prostaglandin and leukotrienes by the COX pathway.[350] Elevated levels of free arachidonate have been found in ischemic/anoxic brain[262,353] and in glutamate-treated slices of hippocampus where it is believed to be due to the activation of cPLA2 by $Ca^{2+}$ entering through the open NMDA receptor channels.[354–357] Arachidonic acid also uncouples mitochondrial oxidative phosphorylation[358] and causes the opening

of the mitochondrial permeability transition pore, a megachannel we will soon meet below which can unleash a lethal cascade of events. Indeed, the brains of mice lacking cPLA2 are less susceptible to ischemic injury.[359] A number of studies have indicated that a glutamate-induced $[Ca^{2+}]_i$ surge causes the translocation to membranes and activation of PKCs which are known to be involved in ROS generation.[360,361] Thus, the ROS that damage cellular proteins, nucleic acids, and membrane lipids collaborate with the excitotoxic glutamate to severely damage and kill neurons.

As if all of this were not enough, an excessively elevated $[Ca^{2+}]_i$ also generates NO (nitric oxide). NO is not highly toxic by itself, but the peroxynitrites produced when it reacts with oxygen radicals such as superoxide are very toxic.[362,363] In neurons, NO is produced by the neuron-specific NO synthase (nNOS), which is a $Ca^{2+}$·CaM-activated enzyme.[363] The surge of $Ca^{2+}$ through continuously stimulated NMDA receptor channels and the resulting $Ca^{2+}$·CaM complexes should and indeed do result in an excessive production of NO in glutamate-treated cultured neurons.[364–369] However, the NO story is rather confusing because NO has been implicated in both neuronal death and survival.[362,370] In cultured neurons, glutamate-induced excitotoxicity has been linked to NO production.[367–372] Furthermore, it has been shown that mice lacking nNOS are resistant to NMDA-induced neuronal damage in vivo,[373] and the neurons in cortical cultures from these mice are resistant to glutamate-induced killing.[373] Other studies could not link NO to neuron killing.[373] NO toxicity could result from its effect on the electron transport system and inhibition of mitochondrial respiration.[363,374,375] NO can reversibly interact with mitochondrial cytochrome oxidase (in the proton-pumping complex IV) and react with superoxide anion to produce peroxynitrite.[363,376–378] Peroxynitrite is known to inhibit both the Mn- and Cu/Zn-superoxide dismutases that sweep up ROS.[379,380] High concentrations of peroxynitrites also inhibit cytochrome c reductase in the proton-pumping complex III of the respiratory chain.[381,382] Moreover, it can also inhibit the plasma membrane glutamate transport mechanism.[383] Thus, NO could kill neurons mainly by inhibiting mitochondrial complexes III and IV and with this the stopping of the spinning-inducing flow of protons through the ATP generators and the consequent conversion of the ATP generators into ATP-destroyers.[384]

The involvement of mitochondria in many neurodegenerative disorders has attracted much research interest lately. Most of the attention is focused on the relationship between the glutamate-induced increase in $[Ca^{2+}]_i$ and mitochondrial $\Delta\psi$. Prolonged activation of NMDA receptors massively increases $[Ca^{2+}]_i$ and kills cultured hippocampal, cortical, and cerebellar neurons.[385–388] The involvement of mitochondria in this neuron killing is indicated by the ability of inhibitors of the electron transport chain to increase the sensitivity of the cells to NMDA-induced $Ca^{2+}$ overload . Thus, hippocampal cells exposed to antimycin A (a complex III inhibitor) and cyanide (a complex IV inhibitor) were more vulnerable to killing by NMDA.[389–390] Inhibitors of the mitochondrial proton-pumping complex I also increased the vulnerability of neurons to murder by NMDA.[391,392] As mentioned above, one of the earliest events in the killing of neurons is the collapse of cytosolic $Ca^{2+}$ homeostasis, also referred to as delayed $Ca^{2+}$ deregulation, DCD, which occurs in neurons, following the overstimulation of NMDA receptors.[321,322,325] We also saw that much of the $Ca^{2+}$ flowing into the cell through the NMDA receptor channels starts being taken up by mitochondria once the rising level of cytosolic $Ca^{2+}$ reaches reaches a set point. This in-rushing $Ca^{2+}$ depolarizes mitochondria and

somehow triggers the production of ROSs,[321,322,325] which, as mentioned above, do many damaging things such as disable the $Ca^{2+}$ pumps in the plasma membrane.[393–396] $Ca^{2+}$ pump failure further increases the cytosolic [$Ca^{2+}$], which in turn increases mitochondrial $Ca^{2+}$ loading and depolarization, ending in the collapse of the mitochondrial $\Delta\psi$.[321,322,325] The combination of $\Delta\psi$ collapse, free radical generation, NO production, and increased ADP/ATP ratio (resulting from the collapse of the $\Delta\psi$ and ATP production) trigger the opening of a megachannel at the contact points between the inner and outer mitchondrial membranes — PTP, the inner mitochondrial permeability transition pore.[321,322,325] PTP is probably the key mediator of the mitochondria's involvement in cell death.[321,322,325] The opening of the PTP allows ions and molecules such as cytochrome c to leak into the cytoplasm and trigger apoptosis.[397–399] Indeed, inhibiting the opening of the PTP megachannel with the immunosuppressent, cyclosporin A, has been shown to prevent or delay mitochondrial depolarization and promote the recovery of $\Delta\psi$ in cortical and cerebellar granule cells.[400,401] This is probably how cyclosporin A prevents or delays neuronal apoptosis in stroke and other neurodegenerative conditions.[402–406]

Mitochondria are believed to play a role in both necrotic and apoptotic deaths of neurons.[407] Necrotic death occurs when the neuronal injury is severe enough to completely collapse mitochondrial function and shut off the ATP supply.[322] When the injury is less severe, although still fatal, mitochondrial depolarization and opening of the PTP will release a flood of apoptogenic agents into the cytoplasm, but the mitochondria will still be able to make enough ATP to fuel the apoptogenic mechanism.[322] $Ca^{2+}$ is involved in both kinds of killing. The big PTP pore is made up of inner membrane proteins such as the adenine nucleotide translocator (ANT) and outer membrane proteins like porin, which is a voltage-dependent anion channel (VDAC) and its associated protein cyclophilin D.[398,408] In fact, the inhibition of pore-opening and the above-mentioned neuroprotective effect of cyclosporin A is due to it preventing the pore from forming by binding to the pore's cyclophilin D component.[398,408] The $Ca^{2+}$-triggered opening of a huge hole in the mitochondrial inner membrane collapses the $\Delta pH$, which uncouples mitochondrial respiration from ATP synthesis, induces mitochondrial swelling, and releases apoptogenic agents such as cytochrome c, AIF (apoptosis-inducing factor), and procaspases into the cytoplasm.[398,408–410] The mitochondrial $Ca^{2+}$ loading, oxidative stress, and ATP shortage that open the big channel appear to collaborate in the killing of neurons in the ischemic brains. There are also strong indications that the collapse of $\Delta\psi$ recruits the apoptogenic, channel-forming Bax protein into the initial pore complex along with a number of other proteins, and that the Bax channel is the conduit through which various apoptogenic molecules escape into the cytosol during apoptosis.[411] Both Bcl-2 and Bcl-$X_L$ protector proteins can bind Bax to prevent megachannel formation and thus prevent $Ca^{2+}$-induced neuronal apoptosis.[411,412] While the cytochrome c flowing out of the mitochondria through the PTP megachannel triggers protein degradations in the cytosol by activating proteases known as caspases, AIF flowing out along with it continues on to the nucleus where it induces the apoptotic destruction of chromatin.[398]

The apoptogenic factors flowing out of the big channels in dysfunctional mitochondria can affect a number of signaling molecules, including protein kinases and transcription factors, many of which are implicated in cell death.[398,413–415] Thus, proteolysis by caspases activates kinases such as PKC-δ, MEKK1, FAK, and PAK2, which are known to promote apoptosis. Furthermore, the cleaved kinase domain of MEKK1 can itself

stimulate caspase activity to further promote apoptogenesis.[416] Caspases may increase neuronal susceptibility to apoptotic death by destroying 'cell survival' proteins such as Akt-1 and Raf-1 kinases.[417,418] The destruction of Raf-1 is serious because it is taken to mitochondria by Bcl-2 where it would normally phosphorylate BAD and thus facilitate the anti-apoptotic function of Bcl-2.[419] Consequently, the destruction of Raf-1 by caspases will prevent BAD phosphorylation and inhibit the protective function of Bcl-2. Suppressing mitochondrial Raf-1 enzymatic activity has also been shown to inhibit the anti-apoptotic activity of Akt.[420] Caspases also hobble the DNA-repair machinery by destroying the DNA-dependent protein kinase, (DNA-PK), and poly (ADP-ribose) polymerase (PARP).[421] A number of cytoskeletal and nuclear proteins such as tau, GAS-2, gelsolin, fodrin, nuclear lamins and the inhibitory subunit of DNA fragmentation factor, U1-70K and NuMA, have been shown to be destroyed by caspases,[421,422] and their destruction is likely to be involved in apoptogenesis. Activation of calcineurin, a $Ca^{2+}$-dependent protein phosphatase (PP2B), by the $Ca^{2+}$ influx can dephosphorylate BAD, which then translocates to mitochondria and promotes apoptosis by forming a complex with Bcl-$X_L$.[423] Indeed, it has been shown that the glutamate-induced $[Ca^{2+}]_i$ increase in hippocampal neurons can activate PP2B and trigger the translocation of BAD to mitochondria and apoptosis.[423]

Many transcription factors are implicated in apoptosis. The transcription factor NF-κB is known to play a role in apoptogenesis in many cells by controlling the expression of genes involved in inhibiting cell death.[424] Thus, inhibition of transcriptional activity of NF-κB has been shown to promote cell death in many cases.[424] Recent studies have indicated that the proteolytic cleavage of NF-κB or its suppressor protein I-κBα by caspases produces dominant-negative fragments that block NF-κB activity.[425,426] Consequently, caspases can promote cell death by blocking the protective effect of NF-κB. Another transcription factor, p53(TP53), appears to play a key role in apoptosis induced by various stimuli including ROS-induced DNA damage and hypoxia.[427] p53(TP53) has been shown to stimulate pro-apoptotic genes such as Bax and silence anti-apoptotic genes such as Bcl-2 thereby shifting the balance towards apoptosis.[427] p53(TP53) also induces p85, a regulator of PI-3 kinase, which has been shown to participate in oxidative stress-induced apoptosis.[427] The activity of p53(TP53) is suppressed by the mdm-2 oncoprotein, which, as we learned in Chap. 2, binds p53(TP53), prevents it from stimulating gene expression, and delivers it to the proteasome shredder.[427] Recently, it has been shown that mdm-2 is cleaved by a specific caspase which stops p53(TP53) degradation and thus stabilizes its apoptogenic function.[428] Caspases also destroy cell-cycle-suppressor proteins such as the E2F1 binding/suppressing retinoblastoma (pRb) 'pocket' protein. The destruction of pRb unleashes E2F1, which stimulates the expression of the apoptogenic p53(TP53), which feeds back and promotes the further destruction of RB by the caspases.[427] Clearly, the creation of the mitochondrial megachannel triggered by an uncontrolled $Ca^{2+}$ surge sets off an impressive cascade of lethal events.[429]

Besides indirectly activating caspases, the surging $Ca^{2+}$ directly activates other proteases — the calpains we met in Chaps. 2 and 3.[430] There are two isoforms of this enzyme, μ-calpain activated by micromolar $Ca^{2+}$ and m-calpain activated by millimolar $Ca^{2+}$, both of which are highly expressed in all parts of the brain.[430,434,435] μ-Calpain appears to predominate in neurons.[430–435] Calpain targets a number of cellular proteins, which includes cytoskeletal proteins (spectrin, MAP2, tubulin), membrane proteins such as

$Ca^{2+}$-channel components, the neural N-CAM cell-adhesion molecule, the EGF and PDGF receptors, and enzymes such as $Ca^{2+}$-ATPase, the CaMKs, and the PKCs,[430,431] which are variously involved in neuronal survival and death. Moreover, both calpains and caspases share many substrates.[431] Unlike the apoptogenic caspases, the calpains appear to be involved in both necrosis and apoptosis.[430,431] There is growing evidence to suggest that $Ca^{2+}$-activated calpain plays a key part in excitotoxicity-induced neuron killing.[431,436] A burst of calpain activity follows glutamate or NMDA treatment of cultured hippocampal and cortical neurons,[437,438] and blocking calpain activity with specific inhibitors protects neurons from excitotoxicity.[438–440] Calpain blockers have been shown to protect neurons against hypoxia-induced death.[441,442] There is some evidence to suggest that only when $Ca^{2+}$ enters through certain ports, such as the NMDA receptor channel, can it activate calpain, which then helps to murder the neuron.[437]

Our tour of the world of brains and neurons must come to an end. Once again, we have seen the many faces of $Ca^{2+}$. We have seen it building brains by driving neuroblast proliferation and the migration of axonal growth cones, operating and individually shaping brains by triggering release of neurotransmitters, but we have also seen it killing neurons and destroying brains.

Now we must go on to the last part of our tour of Calcium Land. We will find this last place very different — yet maybe not so different.

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# 6
# But What About $Ca^{2+}$ and Plants?

*".....$Ca^{2+}$ is, probably, a universal messenger in transducing external stimuli in plants."*

— B.W. Poovaiah and A.S.N. Reddy[1]

*"The $Ca^{2+}$ hypothesis is alive and kicking in plant cells as in other eukaryotic systems."*

— A. Trewavas et al.[2]

Plants don't have muscles to move them around or experience-sculpted brains to program and direct them. Nevertheless, they can sense and respond almost instantly to a wide variety of stresses. And $Ca^{2+}$ signature-surges are the principal agents of their responsiveness.[3] The basic $Ca^{2+}$-signaling mechanisms appeared 2 Ga ago in the first Archeons, long before animals and plants went their separate ways. Therefore, we should expect that many, if not most, plant cell functions should be driven by $Ca^{2+}$, CaMs, calcineurin, the CAMKs, and other signaling mechanisms that are similar or identical to those that drive the many functions of our cells. As we shall now see from these few examples, this is indeed the case.

## $Ca^{2+}$ and blowing in the wind

Despite not having nervous systems, plants, like our bones, have a mechanosensory device(s) that can respond to the strains caused by touching, bending, and shaking by causing structural changes that minimize the strain. The morphological changes caused by touching has been called *thigmomorphogenesis*, and the response to shaking is called *seismomorphogenesis*.[2,3] When woody plants are being rocked by wind, they stop growing upward (which would exaggerate the bending strain produced by a given amount of stress) and redirect their carbohydrate resources to thickening and lignifying their stems to reduce the bending at the stem–root junction. The lack of a need to redirect resources from seed-making to strengthening stems is why seed yields are greater in greenhouse-grown plants that are sheltered from the wind strain.[2]

The ability of touching to trigger $Ca^{2+}$ surges has been demonstrated by engineering *Nicotiana plumbaginafolia* to express apoaequorin (part of a jellyfish's anti-predator luminescent protein aequorin [coelenterazine·apoaequorin]) and then exposing seedlings to coelenterazine to make the complete aequorin $Ca^{2+}$ sensor protein.[3,4] These aequorin-loaded seedlings light up almost immediately when touched or slightly rocked. The

alternating compression and tension caused by touching or rocking trigger sharp gene-activating spikes of $Ca^{2+}$, the amplitudes of which depend on the amount of strain.[3–4] These strain-induced surges of $Ca^{2+}$ from internal stores (possibly from the vacuole, the major $Ca^{2+}$ storage site or maybe from the endoplasmic reticulum)[3,5] trigger a flurry of gene activity, the intensity of which depends on the amount of strain and is responsible for the strain-induced morphogenesis.[2] In *Arabidopsis*, touch-triggered $Ca^{2+}$ spikes stimulate the expression of the so-called TCH genes, some of which encode at least three of the plant's six CaM genes.[3,3a,6–8] The TCH3 gene encodes a novel $Ca^{2+}$-binding protein, which complexes with six $Ca^{2+}$s, instead of CaM's four, and probably does not have the same targets as $Ca^{2+}$·CaM.[6] The mechanoinduced TCH3 protein accumulates at points of maximum strain where cell–wall reinforcement is needed.[7] This accumulated TCH3, responding to the strain-triggered spatio-temporal $Ca^{2+}$ spikes, may stimulate, or collaborate with the product of, another TCH gene, TCH4, which encodes a xyloglucan endotransglycolase (XET).[7,9,10] When expressed, this enzyme would strengthen the cell walls at these strain hot spots by crosslinking the walls' cellulose microfibrils with xyloglucan.[7]

## Calcium and light

Some plants have been found to respond to light red or blue light with changes in the cytoplasmic $Ca^{2+}$ concentration.[3a] $Ca^{2+}$ and CaM are involved in inducing the expression of one of the genes, *gsa*, involved in chlorophyll synthesis in *Chlamydomonas*.[3a] Moreover, external 3–5 mM $Ca^{2+}$ can actually eliminate the need for light to trigger the formation of primary leaves by *Sorghum bicolor* grown in the dark.[3a]

## Cold, drought, and $Ca^{2+}$

A major 'worry' for plants is their water supply. Freezing, drought, or excessive salination of their soil will restrict their ability to pick up and transport water through their vascular systems to their various parts, and a continuing loss of water through the small openings in the epidermis of their leaves, the stomata, becomes dangerous. And they use $Ca^{2+}$ to trigger their responses to a water shortage.

A cold shock (e.g., 20°C→0°C) depolarizes the plasma membrane and causes the influx of $Ca^{2+}$ into the cells of alfalfa, *Arabidopsis*, corn (maize), and tobacco.[3] The shock also activates PLC, which generates $InsP_3$ that causes the release of vacuolar $Ca^{2+}$ to supplement the $Ca^{2+}$ flowing in through the plasma membrane. The $Ca^{2+}$ signature-surge stimulates the expression of various cold acclimation genes (*cas*[15], *cas*[18], *kin*, TCH2, 3, 4) by stimulating the genes for ABA (abscisic acid) synthesis. Of course, the $Ca^{2+}$ surge generates $Ca^{2+}$·CaM, which stimulates among other things L-glutamate decarboxylase, which makes γ-aminobutyric acid (GABA).[3,3a] One of the indirect, possibly ethylene-mediated, effects of the $Ca^{2+}$- triggered ABA surge is the activation of hydrolytic enzymes that break the tethers holding the cells together in the leaf abscission zones, which causes the leaves to fall off (i.e., it causes *apoptosis* because this Greek word actually means the falling of leaves!).

When the roots of a plant in a drought-stricken or excessively saline soil sense the resulting water shortage, there is a burst of PLC activity and through this the

production of $InsP_3$ that triggers the release of $Ca^{2+}$ into the cytoplasm from the internal stores.[3] Obviously, the first thing to do is stop the 'hemorrhage' of the precious water through the leaves' stomata. This is done by the $Ca^{2+}$-stimulated expression of ABA that causes the guard cells regulating the size of the stomata to slam shut.[3] This phytohormone also triggers the cascade of mechanisms leading to the accumulation of osmocompatible solutes and osmoprotectors, promoting dormancy, and perhaps getting rid of leaves.[3]

## $Ca^{2+}$ as a driver of growth and proliferation

One of the earliest, if not the first, indications that $Ca^{2+}$ controls the initiation of DNA replication in plant cells was provided by Setterfield,[11] using slices of quiescent Jerusalem artichoke (*Helianthus tuberosus*) tuber. The quiescent cells in these slices can either start expanding when activated by an auxin BTOA (benzthiazolyloxyacetic acid, a β-indoleacetic acid analog) and kinetin or start making DNA after a 20-h lag period and divide without expanding. It is $Ca^{2+}$ that determines whether the cells will proliferate or just expand. In fact, the responses are exactly like the responses of hepatocytes to partial hepatectomy in normal and thyroparathyroidectomized rats that we talked about in Chap. 2. If the tuber cells are stimulated by addition of BTOA and kinetin in a $Ca^{2+}$-free medium, they will expand by 40% without initiating DNA replication. However, if the medium contains $Ca^{2+}$ (30 meq/L) the cells can start making DNA between 20 and 24 h after adding the hormones, but they will not expand.

$Ca^{2+}$ surges are involved in starting the growth and proliferation of quiescent cambial cells in the beech tree (*Fagus sylvatica*).[12] As the tree emerges from its winter quiescence, there are large transient $Ca^{2+}$ surges in the cambium and phloem, which probably trigger events leading to cambial cell proliferation, with the production of secondary phloem and xylem and an increase in the stem's girth.[12]

Another example of $Ca^{2+}$ and cell cycle starting is provided by the proliferogenic action of lipo-chito-oligosaccharide Nod (nodule) factors from bacterial *Rhizobium* species on root cells of leguminous plants. These factors trigger cytoplasmic and perinuclear $Ca^{2+}$ surges by directly stimulating the entry of $Ca^{2+}$ into the root hair cells through the cell wall and membrane and (or) by activating surface receptors which: (1) causes the root hair to curl and grab the bacterium by disrupting actin microfilaments; (2) switches on $G_1$ phase-specific, cyclin-dependent protein kinase(s) to stimulate the proliferation of cortical cells; and (3) through these changes causes the ultimate formation nitrogen-fixing nodules on the root hairs.[13–15]

Very little is known about the $Ca^{2+}$ spikes and oscillations — the $Ca^{2+}$ songs — and the $Ca^{2+}$-binding signal proteins that we suspect are involved in triggering the key transition points in plant cell cycles. However, we can see some light at the end of this tunnel of ignorance! One hint of $Ca^{2+}$ being the same major player in driving plant cell proliferation as it is in animal cells is the high level of CaM mRNA in proliferatively active root and shoot meristems.[16] But higher plants do some things differently. Their predominant cell cycle-driving $Ca^{2+}$-binding/activated signaler(s) is not CaM. Instead, they use the ingeniously designed CaDPKs ($Ca^{2+}$-dependent protein kinases) that have a catalytic domain that is homologous to the mammalian $Ca^{2+}$·CaM-activated CaMKII plus a built-in $Ca^{2+}$ sensor — a CaM-like domain.[3a] In other words, these enzymes can sense and

respond to $Ca^{2+}$ directly without having to wait for $Ca^{2+}$·CaM to appear. A proliferogen such as the auxin, 2,4-dichlorophenoxy acetic acid, but not the anti-proliferative ABA, stimulates the expression of an alfalfa CaDPK gene.[16] And CaDPK mRNA peaks in alfalfa cells first in the S phase and again in the mid-$G_2$.[16] Thus, it is probably safe to assume that the starting of a cell cycle and the initiation of DNA replication in plant cells are triggered by the same $Ca^{2+}$ spikes and oscillations that we saw in the animal cells of Chap. 2. There is already much support for a surge of $Ca^{2+}$ from spindle-associated stores triggering anaphase.[17-23] $Ca^{2+}$ is also probably involved in cytokinesis but not as it is in animal cells. In higher plants, cytokinesis starts when the $Ca^{2+}$ surge crashes at the end of anaphase and spindle interzonal microtubules, actin micofilaments, Golgi-derived vesicles loaded with cell wall matrix polysaccharides, and endoplasmic reticulum collect into a cell-partitioning, cell wall precursor, the phragmoplast, instead of the cytoplasm simply being pinched in two by the $Ca^{2+}$-driven tightening of a subcortical actomyosin belt as in the wall-less animal cells.[17-23] $Ca^{2+}$ probably drives the delivery of components to the phragmoplast construction site, the formation of the tubulo-vesicular scaffold, and the correct positioning of the forming structure.[21]

It is also becoming increasingly certain that the plant cells drive their cycles with stage-specific Cdk·cyclin kinases, which do the same jobs as the homologous cycle engine kinases of animal cells.[24-26] Also, despite the once universally held belief that higher plants do not use cyclic AMP-dependent control mechanisms, it is now becoming ever clearer that they have adenylyl cyclase and use it and its cyclic AMP product to do key things.[27-30] Indeed, it seems that a small $G_1$ and a larger S-phase cyclic AMP surges work along with $Ca^{2+}$ and the Cdk·cyclin kinases to initiate DNA replication and prepare for mitosis in plant cells such as BY-2 tobacco cells just as they do in animal cells.[27]

One of $Ca^{2+}$'s many jobs in both plants and animals, which dates back to the first pre-eukaryotes, is operating the motor the cell uses to crawl toward its targets. A striking example of this in plants is pollen tube growth. When a pollen grain lands on the stigma of a pistil of a flowering plant, it will start producing a tube, if it is recognized as being compatible and receives appropriate starting signals.[31,32] Then, the tube, with its precious cargo, two haploid male nuclei, pushes between the stigma cells and moves through the transmitting tract of the pistil's style at a rate that can be as high as 1 cm/h. As with a neuron's axonal growth cone, the direction-sensing engine (or 'brain') of the pollen tube is in its tip.[32] The navigation of the tip is directed by signals generated by the interaction of the tube with specific stylar guidepost glycoproteins. However, from our perspective the most interesting part of the motor's driving mechanism is the tube's apical $Ca^{2+}$ gradient produced by $Ca^{2+}$ surging through channels at the tip of the tube.[15,33] Also concentrated at the tip is another part of the driving mechanism, a CaDPK which is undoubtedly a mediator of the $Ca^{2+}$ signal surges.[3] The driving ability of the $Ca^{2+}$ pulses is dramatically demonstrated by a release of intracellularly 'caged' $Ca^{2+}$ at one side of the tube apex, causing a redistribution of the CaDPK to the release site, the creation of a new tip, and the reorientation of the tube's movement.[15,33] It should be noted in passing that exactly the same apical $Ca^{2+}$ gradients and pulses drive the extension of root tips.[15]

The tip of the pollen tube, with its $Ca^{2+}$ pulses, eventually arrives at the ovule, crawls along the ovule's funiculus or stalk, and, guided by appropriate signalers, passes through the micropyle and into embryo sac containing the egg cell.[32] The tip of the tube ruptures and releases the two haploid male nuclei. One of these nuclei fuses with the egg. The

other fuses with the primary diploid endosperm nucleus to form the triploid endosperm cell, which will start dividing to form the endosperm that will, in turn, accumulate 'yolk' for feeding the germinating seed. The fusion of a sperm cell with an animal egg cell triggers a $Ca^{2+}$ 'tsunami' that spreads over the egg from the fusion site to start the proliferation of the zygote. A similarly spreading $Ca^{2+}$ wave is triggered by the fusion of a *Zea mays* egg with a male nucleus.[34] It is probably safe to predict that a $Ca^{2+}$ transient also triggers the division of the endosperm nucleus after the fusion of the other male nucleus with the primary endosperm nucleus.

## Conclusion

We, and probably the reader, find this venture into the plant world rather cursory. We shall not venture further into this wonderful world mainly because of our all too obvious lack of credentials and most of all because the field has only just started to emerge. However, we have at least touched the buds that will very soon burst into a mass of exciting data, telling us how $Ca^{2+}$ controls the various functions of higher plants. Given the vast antiquity of the ion's hold on eukaryotic cells, the themes will be generally familiar, yet at the same time fascinatingly different from those in the animal world.

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# 7
# Summary

Our brief tour of the highlights of the parts of the current $Ca^{2+}$ world concerned with cell cycles, differentiation, and death has ended. We have seen the $Ca^{2+}$ oscillations that trigger protein synthesis and the delivery of transcription factors from the cell membrane and cytoplasm to the nucleus and that activate transcription factors already attached to their target genes' promoters in the nucleus. The products of these genes started the first wave of proliferogene activities that, in turn, started the parade of cyclin-dependent protein kinases beginning with Cdk4·cyclin D, which inactivated the gene transcription-suppressing Rb protein, initiated the buildup of the cation channels, receptors, and the autocrine/paracrine second signaler, such as IGF-I or IL-2, to use these devices. We saw the emergence of Cdk4·cyclin D's successor, Cdk2·cyclin E, that continued the inactivation of Rb family members to enable E2F·DP-1 transcription factor to stimulate the expression of key DNA replication enzymes. Then there was the downregulation of Cdk2·cyclin E and the cyclic AMP- and substrate adhesion (i.e., integrin signaling)-dependent emergence of Cdk2·cyclin A, which collaborates with another train of $Ca^{2+}$ oscillations triggered by autocrine IGF-I and enhanced by c-Myb to activate the huge DNA replication factories attached to the chromosomal replication origins on the nuclear matrix. When all of the chromosomes were replicated and after the $G_2$ delay, there was the $Ca^{2+}$ spike at the nuclear envelope that activated the first of the two emerging mitotic cylins, Cdk1·cyclin A, which triggered the chromosome condensation and nuclear envelope breakdown of mitotic prophase and then crashed to leave the rest of the job to Cdk1·cyclin B. This was followed by another $Ca^{2+}$ spike when the chromosomes had lined up on the mitotic spindle, which, by activating the anaphase-promoting polyubiquitination-proteolysis mechanism that destroyed Cdk1·cyclin B, enabled the licensing of the sister chromatids for future replication, the removal of the residual DNA strand tangles holding the sister chromatids together, the destruction of the protein glues that also held the sisters together, and started the motors that moved the sister chromosomes to opposite spindle poles. We saw the cell divide when yet another, but this time more prolonged, $Ca^{2+}$ surge drove the contraction of an equatorial belt of actomyosin filaments that pinched it into two daughters.

We then traveled to the skin where we saw $Ca^{2+}$ drive the cycling of keratinocytes attached to the epidermal basement membrane and a $Ca^{2+}$ surge trigger the spinous program when the basal keratinocytes lost their β1 integrins and with them their cycle-supporting, differentiation-suppressing signals and lifted off the basement membrane. We learned of the upwardly directed epidermal $Ca^{2+}$ gradient, which near its top in the granular layer stimulates the divalent cation receptors on the cell's surface to signal the cell to generate an internal $Ca^{2+}$ surge large enough to start the final cascade of differentiation/apoptosis-like events that transform the cell into a dead cornified shell packed with

keratin macrofilaments. Here we learned one of the most important features (established mainly here in Ottawa) of epithelial cell carcinogenesis: loss of responsiveness to $Ca^{2+}$ signals that would normally stop cycling and trigger differentiation and apoptosis (diffpoptosis).

When we moved from the skin to the colon, we again saw $Ca^{2+}$ driving the cycling of the transit-amplifying progeny of stem cells in the depths of the colon crypts. We probed the possibility of there being an upwardly directed $Ca^{2+}$ gradient like the gradient in epidermis which activates the divalent cation receptors on the colon cells' surfaces that send signals that drive, or at least substantially contribute to the driving of, the differentiation and ultimately apoptotic killing of the cells in the upper regions of the crypt and out on the flat mucosal surface. Here too an early loss of responsiveness to the $Ca^{2+}$ signals commanding the cells to stop cycling, differentiate, and then die by apoptosis is a key part of the progression to malignancy. We then saw how $Ca^{2+}$ might operate the brakes and accelerator of the engine that drives the axon growth cone as it advances toward its target. We also learned that the ion seems to similarly control the engine that drives the plant pollen tube toward its target egg cell. We saw $Ca^{2+}$ and its client signal proteins driving the release of neurotransmitters and recording memories, but we also saw that given the right circumstances it can be an efficient neuron killer. Finally, we learned to look at plants in a very different way. We learned that although plants do not have brains and muscles for fight and flight, they nevertheless can rapidly respond with growth-altering $Ca^{2+}$ signals to light, mechanical strain and various environmental challenges such as cold and drought. Indeed, the story of $Ca^{2+}$ in plants is only now starting to be told.

Unfortunately for us, $Ca^{2+}$ is a very busy ion with lots of different targets and mediators scattered throughout the animal and plant cell. But we can at least make out the dim, but tantalizing, outlines of the ion's diverse roles in cell cycles and diffpoptosis. However, the tools are now being invented to accurately map in cellular space and time the $Ca^{2+}$ spikes, waves, and oscillations that start the several stages of the cell cycle and diffpoptosis. Clearly, these new more sophisticated tools will give us insights into the awesome networks used by $Ca^{2+}$ in its roles of cell cycle driver, differentiator, and killer.

# Index


## A

Abscisic acid (ABA) 230–232
Acetyl-CoA carboxylase 105
ACFs (aberrant crypt foci) 154–157, 159, 160
α-Actinin 78, 90, 148
Adenoviral E1 protein 76
Adenylyl cyclase 93, 94, 96, 111, 152, 155, 181, 189, 232
Adenylyl cyclase, type1 180, 188
ADSP (activity-dependent synaptic plasticity) 179
Aequorin 229
AIF (apoptosis-inducing factor) 199
AKAP (A-kinase-anchoring protein) 21
Akt-1 kinase 200
Alzheimer's disease 31
AMPA (glutamate receptor channels) 183, 184, 186, 188–190, 196
Amphiregulin 108, 151
Anaphase 7, 15, 18, 25–30, 43, 47, 60, 86, 232, 237,
Anaphase-GO! signal 27, 29, 60
Annexin I 104
Annexin II 57, 88
APC (anaphase-promoting complex) 18, 27, 29, 30, 60
APC (adenomatous polyposis coli) 78, 142, 148, 150, 152, 156–159, 161, 162
APC·p55CDC(Cdc20)anaphase-promoting complex 29
Apex cherts 2
AP-1 heterodimer, motifs 90
*Aplysia* neurons 183
Apoaequorin 229
Apoptosis 7, 11, 19–22, 49, 50, 75, 77–79, 81, 83, 85, 86, 88, 96, 103, 107, 109, 114–116, 142, 143, 145, 146, 148, 150–152, 154–156, 160, 161, 177, 179, 193–195, 199–201, 230, 237, 238
*Arabidopsis* 230
Archeons 4, 229
ATM (ataxia telangiectasia) 22, 23
ATP–ADP transporter 49
ATPase (synthase) 4, 55, 56, 104, 150, 192, 194–197, 199, 201
ATP synthase (mitochondrial) 195, 199
Auxin 231, 232
Axin protein 78, 148, 150, 156

## B

BAD protein 19, 21, 49, 78, 148, 152, 159, 200
BAFs (bulk adduct formers) 153, 154
Basal cell(s) 11, 17, 75–80, 84, 86, 87, 89, 92, 93, 95, 96, 101, 105, 107, 109, 110, 112, 114, 141, 144, 146, 148, 151
Basal cell carcinoma 111–114
Bax protein 23, 77, 79, 83, 86, 88, 96, 152, 159, 199, 200
Bcl-2 19, 23, 49, 56, 77–79, 85, 86, 88, 89, 96, 107, 113–115, 143, 146, 148, 152, 155, 159, 160, 179, 199, 200
Bcl-w protein 160, 161
Bcl-$X_L$ 77, 79, 83, 85, 86, 88, 89, 199, 200
Bcl-$X_S$ 152
bFGF 21, 80, 94, 108, 115
Biosymbiogenesis (endosymbiosis) 5
B-*myb* gene 17, 18
B-Myb protein 14, 18, 55

# C


$Ca^{2+}$-ATPase pumps 104
$Ca^{2+}$-binding proteins 6, 58
CACPC (Common Ancestral Community of Primitive Cells) 3, 5
CaDPK ($Ca^{2+}$-dependent protein kinases) 187, 231, 232
Calbindin 147, 194
Calcineurin 48, 178, 179, 188
Calmodulin 7, 29, 30, 43, 48, 53, 55, 57, 58, 95, 104, 194
Calpain 14, 19, 56, 59, 60, 104, 199, 200
"Cambrian Explosion" 4
CaM genes 230
CaMKII 48, 49, 57–60, 177, 181, 187–189, 191, 194, 197, 231
CaMKIV 48, 89, 98, 185, 187, 189
CA1 neurons 186–188
$Ca^{2+}$ puffs, sparks, waves 5
$Ca^{2+}$ pump 7, 55, 81, 85, 104, 107, 183, 185, 196, 197, 199
Caspases 106, 199–201
α-Catenin 78, 90, 148, 150
β-Catenin 19, 20, 49, 76–79, 86, 89, 90, 107–110, 112, 113, 142, 148, 150, 152, 156, 158, 159, 161, 162
γ-Catenin 89, 108
CBP (CREB-binding protein) 78
CCT (cytosolic chaperonin) 16
Cdc6 14, 23, 25, 30
Cdc25A 13, 14, 16, 49, 57, 148
Cdc25C 13, 28, 49, 60
Cdc7·Dbf4p(ASK) protein kinase 24
Cdk1·cyclin A 15, 27, 30, 60, 61, 86, 95, 237
Cdk1·cyclin B 15, 27–31, 49, 60, 61, 86, 95, 237
Cdk2·cyclin A 15, 18–27, 30, 76, 86, 87, 106, 109
Cdk2·cyclin E 13–24, 76, 86, 95, 106, 111
Cdk4·cyclin D 12–17, 22, 26, 76, 86, 110, 111, 148, 157
Cdk4·cyclin D2 13
Cdk7·cyclin H 13, 17, 111
CDKs (cyclin-dependent protein kinases) 11, 13–15, 20, 25, 26, 28, 31, 43, 58, 59, 80, 86, 87, 95, 108, 145, 149, 160, 177
CEA (carcinoembryonic antigen) 159
C/EBPβ protein 89, 100, 107
CENP-E 28, 29
Centriole 7
Centromere 27–29, 60
Centrosome 15, 28
CEP (centromere-binding proteins) 28
c-Fos 45, 48, 100
c-Ha-*ras* protein 108
Chinese hamster ovary cells 26, 56
*Chlamydomonas* 230
Chloroplasts 6
Choanoflagellates 4
Cholesterol sulfate 92, 105
CHOP protein 89
Chromosome condensation 237
Chromosome, licensing 30, 48, 60, 61
c-Jun 46, 48, 50, 100–102, 148
CKI (cyclin-dependent kinase inhibitors) 87
Clay crystals 1
CLED protein (calcium-linked epithelial differentiation protein) 89, 99
CLSP protein (calmodulin-like skin protein) 104
c-Myc 13, 14, 19, 21, 45, 46, 48–50, 55, 76–79, 86, 87, 97, 108–110, 115, 141, 142, 145, 148–151, 155, 156
c-Myc·Max 13, 45, 79
CO 1
$CO_2$ 1, 3–5
Coelenterazine 229
Cohesins 26, 28, 29, 60
Cold acclimation genes 230
Colon cells 11, 54, 78, 83, 145–147, 150, 151, 153–162, 177, 193, 238
Colon crypts 141, 142, 146, 154, 157, 158, 238
Condensin (phospho-condensin) 28, 59, 60

Congression 28
Connexin (Cx-26, -31.1, -43) 86, 92, 113
Corneocytes 75–77, 81, 85, 96, 103, 105, 106, 146
Cornification 84, 102, 103
Cornified envelope 83, 84, 91–93, 97, 99, 102, 103, 105, 106
COX (cyclooxygenase) pathway 197
CpG/GpC islands 26, 153
CREB (cyclic AMP response element-binding transactivator) 20, 21, 48, 78, 98, 190
CREMτ (cyclic AMP response element modulator) 20, 21, 51
Cripto 155
CtBP (C-terminal TATA box-binding protein) 75, 150
Cyanobacteria 2, 3, 6
Cyclin A 15, 18–22, 24, 25, 27, 29, 30, 49, 51, 53, 55–58, 61
Cyclin B 15, 27–31, 49, 58, 61, 86, 237
Cyclin D1 12–15, 17, 19, 31, 49, 76, 86, 90, 109, 110, 142, 145, 148, 155, 157
Cyclin D3 14, 16, 51
Cyclin E 13–24, 27, 31, 51, 53, 56, 57, 60, 76, 79, 86, 95, 106, 111, 237
Cyclin H 13, 17, 48, 111
Cystatin A 92, 102
Cytochrome c 23, 198, 199
Cytochrome P450 197
Cytokinesis 232

## D

Dbf4p (ASK) protein 24
DCC protein 88, 151, 161, 177
Delta 1 protein 75, 85, 86
Dermal papillae 75
Desmosomes 86, 90, 93, 99, 105, 106, 150
DHFR (dihyrofolate reductase) 17
Diacylglycerol 46–48, 54, 79, 93, 98, 156, 158, 185, 186
Diffpoptosis 7, 49, 61, 75, 80, 81, 83–90, 92, 96, 97, 99, 100, 101, 103, 104, 106–108, 110, 113–115, 141–143, 147, 148, 150, 151, 155, 177, 193, 238
1,2-Dimethylhydrazine 158
DNAase I 106
DNA fragmentation factor 200
DNA ligase 1, DNA primase 24, 25
DNA-MTase (5-cytosine DNA methyl transferase) 26, 153, 154, 157, 161
DNA-PK 22–24, 200
DNA polymerase-α 14, 25, 57 88
DNA polymerase-δ 14, 57, 87, 88
DNA polymerase-ϵ (Pol-ϵ) 24

## E

EcaBP (epidermal $Ca^{2+}$-binding protein) 75, 81, 86, 89, 147
E-cadherin 76, 78, 84, 89, 90, 93, 108–110, 114–116, 142, 148–140, 152, 156–159, 161
EC (enterochromaffin) cells 141
EDC (epidermal differentiation gene complex) 90–92, 102
E2F1 12, 14, 16, 17, 19–21, 31, 49, 86, 148
E2F3 12, 14, 16, 17, 19
E2F·DP-1 14, 16–18, 86, 237
EGF 45, 47, 50, 53, 78–80, 94, 97, 108, 109, 115, 145, 147, 150, 151, 155, 201
Eicosanoids 79, 99
eIF-4A 44, 46
eIF-4E 13, 44, 45
Embryo sac 232
Endometrial cells 7
Endonuclease 6, 103, 114, 193
Endoplasmic reticulum–mitochondrial clusters 185
Endosperm 233
Epidermal Langerhans cell 75
*Escherichia coli* 3
Excitotoxicity 193, 194, 196, 198, 201
Exocytosis 104, 182, 190, 191

## F

Facilitation 180–183
F-actin 78, 89, 90, 148
*Fagus sylvatica* 231
FAK kinase 76, 79, 83, 87, 199
Farnesyl pyrophosphate synthase 105
Fatty acid synthase 105
Fecal water 146, 160
Filopodia 178, 179
Fos B 101
Fra-1, -2 90, 100, 101, 148
Fyn kinase 89, 99, 190

## G

Gadd45 114
Gap junctions 92, 102, 107, 108, 110, 113, 114, 144, 160
*gas*1 gene 79
GCDER, Great Calcium-Driven Eukaryotic Revolution 1, 4, 7
Glial cells 177, 180, 196
GluR1 subunit of AMPA receptor 188
Glutamate neurotoxicity 196
Glutamate receptors 179, 181, 183, 184, 195–197
Granular cells 77, 81, 91, 97, 99–102, 104, 105, 107, 116
Greenhouse warming 1
Groucho protein 78, 150
Growth cone 88, 177–179, 193, 201, 232, 238
GSK-3β (glycogen synthase kinase-3β) 19, 20, 78, 89, 148, 150, 156, 159
Guard cells 231

## H

*Helianthus tuberosus* 231
Helicase I 25
Helicase IV 25
Hepatocytes 14, 20, 53–55, 57, 231
Hippocampal neurons 177, 188, 189, 200
Hippocampus 184, 187, 195, 197
hMLH1 gene 161
hMSH2 154–156
Horny envelope 81, 83, 89, 90, 102–104, 107
Hot smokers 2
Housekeeping genes 23, 24, 26
proteins 44, 45
5-HT 141
$5\text{-HT}_{1P}$ 141
Humoral hypercalcemia of malignancy 111

## I

ICAM-1 105
ICE-like proteases (caspases) 56, 85, 88
ICER (CREB, CREMτ inhibitor) 21
Id-1, -2, -3 (inhibitor of differentiation or diffpoptosis) 83, 86, 87
IGFBP-6 92
IGF-I 23, 45, 51, 55, 56, 77, 80, 86, 92, 96, 97, 148, 155, 237
IGF-I receptor 23, 51, 56
IL-1α 105, 108
IL-2 2, 13, 48, 234
IL-6 45, 108
IL-8 105
IL-10 105
ILK (integrin-linked serine/threonine kinase) 19, 20, 49, 56, 76–79, 81, 83, 86–89, 96, 107, 109, 147, 148, 159
Integrin-$\alpha_2\beta_1$ 75
Integrin-$\alpha_3\beta_1$ 75
Integrin-$\alpha_6\beta_4$ 75, 109
Interferon-γ 105
Interstellar cloud 1
Intrakine function (PTHrP) 53, 77, 86, 89, 93–97, 99, 100, 101, 102, 106, 107, 111, 116, 151, 152, 155, 179
Involucrin 83, 85, 87, 90–93, 95, 97, 99, 100–107, 109, 110

IQ domain 178, 179
Iron pyrites 1
Isoproterenol 53, 93

## J

Jun B 100–102
Jun D 50, 90, 100, 101

## K

Kainate receptors 183, 196
Kalinin 75
Keratin 1 83–85, 89, 90, 95, 99, 100, 101, 102, 106
Keratin 5 75, 84, 87, 90, 99
Keratin 10 83–85, 89, 90, 95, 99, 100, 102, 106
Keratin 14 75, 84, 87, 96, 99
Keratinocytes 11, 49, 54, 61, 75, 77, 80, 81, 83–85, 87, 88, 90, 92–97, 99–102, 106, 107, 109–111, 113–115, 146, 147, 150, 152, 158, 177, 193, 237
Keratohyalin granules 102–104, 107
KGF 77, 80, 86, 88, 89, 96, 97, 99, 105
Ki-67 31, 148
Kinesin 28
Kinetin 231
Kinetochore 28, 29, 60
Ki-Ras 108, 156, 159
KRP (kinesin-related protein) 28–30

## L

Lamellar bodies 81, 92, 99, 102, 104–106
Laminin 75, 151, 161
Lamins 200
LCA (Last Common Ancestor) 3
LDCVs (large dense-core vesicles) 190
LEF/TCF transcription factor 19, 78
Leukotriene B4 54
LGCCs (ligand-gated $Ca^{2+}$ channels) 184, 193, 196, 197
Lipid barrier 92, 93, 105
Loricrin Ω-loops 106
Loricrin 102
LTD (long-term depression) 184, 187, 188
LTP (long-term potentiation) 177, 184–190
L-type $Ca^{2+}$ channels 95, 179

## M

MAG glycoprotein (myelin-associated glycoprotein) 177
Mammary cells 7, 20, 55, 88, 89, 97
MAP kinase 46, 50, 52, 78, 79, 159
MARCKS protein 98
MCMs 23–25, 30, 58, 88
*mda*-6 gene 22
Mdm2 19, 22, 49, 157
Metaphase 7, 27, 28, 30, 43, 86
mGluR1 receptors 186, 187, 195
$Mg^{2+}$ (NMDA channel) plug 186, 189, 197
Micropyle 232
Mismatch repair genes 76
Mitochondria 80, 81, 86, 88, 103, 104, 106, 180, 183, 184, 193–200
Mitochondrial $\Delta\psi$ 49, 195, 198, 199
Mitochondrial ANT (adenine nucleotide translocator) 199
Mitochondrial permeability transition pore (PTP) 23, 49, 183, 198, 199
Mitochondrial ΔpH 195, 199
MNNG (*N'*-methyl-*N'*-nitrosoguanine) 153
MNU (*N*-nitrosourea) 153
mSos (adaptor protein) 46, 78, 79

## N

NAD(P)H oxidase 114
$Na^+/H^+$ exchanger (mitochondrial) 195
$Na^+/K^+$-ATPase 150
N-CAM 151, 201
Neonatal hepatocytes 20
Netrin-1 151, 161, 177

Neurofibrillary tangles 31
Neurogranin 31
Neuromodulin (GAP-43) 179, 181, 187
Neurotrophic factors 31
NF-κB transactivator 21, 45, 200
*Nicotiana plumbaginafolia* 229
NIH3T3 cells 49, 51
NLS (nuclear localization sequence) 94, 95
NMDA-induced $Ca^{2+}$ overload 198
NMDAR, NMDA receptor channels 179, 181, 183–186, 188–190, 196–198, 201
nNOS (neuron-specific nitric oxide synthase) 198
Nod factors 231
NOS (nitric oxide synthase) 181
NO synthase 180, 198
Notch receptor 85
NR1 subunit of NMDAR 189, 197
NR2A subunit of NMDAR 189
NR2B subunit of NMDAR 189
N-type $Ca^{2+}$ channels 192
Nuclear matrix 11, 15, 16, 18, 24, 57, 237
Nuclear pores 16, 21, 55, 56

## O

ODC 45
Oöcytes 26
ORC 25, 30, 88
Origins (replication) 2, 11, 24, 58, 60, 79, 86, 88, 177, 237
Ovule funiculus (stalk) 232

## P

PAKs (p21-Activated Kinases) 19
Pancornulins 104
$p19^{ARF}$ 19, 31, 156, 157
PARP (poly[ADP]ribose polymerase) 24, 200
Parvalbumin 194
$p21^{Cip1/WAF1}$ 13, 19, 22, 31, 49, 55, 87, 149, 154, 155
PCNA 14, 22, 24–26, 31, 57, 87, 88
PDGF (plate-derived growth factor) 15, 44–46, 55, 78, 79, 94, 108, 155, 201
PDK-1 (phosphoinositide-dependent protein kinase-1) 78, 79, 89
Pericryptal fibroblasts 144, 145
Permeability barrier 76, 80, 81, 85, 87, 92, 93, 104, 105
PH domain (pleckstrin homology domain) 76
Phospholipase 6, 46–48, 50, 106, 193
Phospholipase A2 99, 197
Phospholipase C, PLC 52, 59, 94, 98, 147, 180, 185–187, 194, 230
Phragmoplast 232
*Pikaia* 4, 93
PI-3K kinase 79, 108
$p15^{INK4b}$ 87, 111, 149
Pistil 232
PKA catalytic subunit 20, 21, 51, 54
PKAI (cyclic AMP-dependent protein kinase I) 50, 148, 158, 159
PKAII (cyclic AMP-dependent protein kinase II) 148, 158, 159
PKB/AKT 19, 21
PKB (protein kinase B) 19, 21, 76, 78, 79, 81, 83, 85–89, 107, 109, 148, 159
PKC(s) 47, 48, 50, 52, 54, 56, 75, 79, 90, 93–95, 97, 98, 100–103, 106, 110, 111, 114, 157, 158, 178, 179, 181, 185–187, 189, 191, 196–198, 201
-α 47, 100, 101, 155, 158
-βI 47, 155, 158
-βII 47, 60, 155, 156, 158
-γ 47
-δ 47, 79, 100, 109, 199
-ϵ 47, 79, 100
-ς 79
-η 47, 79, 100
-θ 47
-μ 47
$p27^{Kip1}$ 12–14, 16, 44, 87, 106, 107, 109, 111, 149
Plasticity 7, 177, 179, 180, 184

PLC-β 95
-β1 91, 97
-β2 97
-γ1 54, 79, 98
-δ1 97
PNA, peptide nucleic acid 2
Pollen tube 232, 238
Polyps 153, 154, 156, 160
Polyubiquitination 13, 19, 21, 29, 60, 237
Prb 14, 87, 200
Precambrian Pilbara 2
Profilaggrin 91, 92, 97, 101–103, 107
Pronuclei 7
Prophase 15, 26, 27, 30, 31, 43, 47, 59, 61, 86, 237
Prostaglandins 99, 197
Proteasome 13, 14, 16, 18, 19, 21, 27, 29, 49, 60, 78, 86, 87, 89, 148, 150, 156, 200
Proteinase 6, 115
Protein phosphatase 1 20, 187, 188
Protein phosphatase 2A 20, 60
Proteobacterium (α) 6
Protocells 1–3, 6
Protochordate 4
Prozak 141
PSD95 (postsynaptic density-95) 188
*Ptch* ("patched" gene) 112, 113, 156
PtdIns-3K 19, 46, 49, 51, 76, 79, 86–88, 107, 109, 110, 147, 155, 159
PtdIns(3,4,5)$P_3$ 19, 76, 79, 87, 89, 108, 147
PTH/PTHrP receptor 53, 93, 95, 96, 111, 152, 155
PTHrP 53, 77, 86, 89, 93–102, 106, 107, 111, 116, 151, 152, 155, 179
p53(TP53) 12, 19, 20, 22, 23, 31, 55, 87, 111, 114, 143, 148, 154, 156, 157, 160, 161
Pyrophosphates 1, 3

## R

Raf-1 kinase 46, 159, 200
Ran/TC4 27, 28
Ras protein(s) 79, 88, 99, 108, 109, 156, 159, 160
RCC1 27, 28, 59
Replication factories 11, 13, 15, 16, 18, 19, 22–25, 28, 30, 54, 56–58, 79, 87, 88, 108, 177, 237
Replication origins 2, 11, 58, 60, 79, 86, 88, 177, 237
Respiratory chain complexes 198
RF-C (replication factor C) 24, 25, 57, 88
*Rhizobium* 231
Ribosomes 46
Ribozymes 2
RI (type I PKA regulatory subunit) 148
RII (type II PKA regulatory subunit) 20
RLF-B (licensing factor) 23, 25, 88
RNA 2, 3, 21, 25, 44, 45, 76, 94, 95, 106
RNA polymerase I 13, 45
RNA polymerase II 12, 13, 16, 17, 49, 78
RNA polymerase III 13
ROS (reactive oxygen species) 6, 193, 197, 198
R (restriction) point 16
RRP (readily releasable pool) 190–192
RyR (ryanodine receptor/channel) 55, 147, 194

## S

S100A4 161
S100A6 92, 161
*Saccharomyces cerevisiae* 7, 20
SCCE (stratum corneum chymotryptic enzyme) 103
Securin 29, 60
Seismomorphogenesis 229
SERCA (sarcoendoplasmic reticulum $Ca^{2+}$ ATPase) 55, 194–196
Serine palmitoyl transferase 165
Serotonin 141, 190
Shc adaptor protein 46, 78

SH2 domain 148
pockets 45, 46, 76, 78, 79
SH3 46, 78, 79, 148
Smad 2, smad 4 proteins 161
Small intestinal crypts 144, 145
Smokers (deep sea) 2
Smo protein ("smoothened" protein) 112
SNAP-25 192
*Sorghum bicolor* 230
Sp1 17
Sperm 7, 233
Spindle 18, 28–30, 60, 114, 232, 237
Spinous cells 77, 78, 80, 84, 92, 94, 96, 97, 100–102, 105
S6 protein (kinases) 44, 46
SPRR (proteins) 90–93, 106
S100 proteins 89, 91, 92, 161
Squalene synthase 105
Squamous cell carcinoma 108, 111, 114
Src kinase 187, 190
SSVs (small synaptic vesicles) 190
STE (short-term enhancement) 180, 182, 183
Stigma 232
Stomata 230, 231
STOP protein 28
Style 232
Sulfomucins 146
Superoxide dismutases 198
Swiss albino 3T3 cells 51, 54, 56
Synapsin 191
Synaptobrevin 192, 193
Synaptotagmin 192, 193
Syntaxin 192, 193
Synthesomes 24, 25, 30, 88, 108

## T

Tau protein 31
T51B rat liver cells 54, 56, 57, 58
TCH gene 230
Telomerase 76, 77, 88, 115, 148, 156
Telophase 30, 60, 61
TERT (telomerase catalytic subunit) 148, 156
TFIID 17
TFIIH 13, 17
TGF-α 47, 76–80, 86, 96, 105, 108, 109, 115, 145, 147, 150, 151, 155
TGF-β1 83, 87, 149, 151, 155, 161
TGF-β2 83, 87, 92, 95, 149, 151, 155, 161
Thapsigargin 55, 85, 194, 195
Thigmomorphogenesis 229
Thyrocytes 20, 50–53
TNF-α 105
Tonofibrillar bundles 84
Tonofilaments 99
Topoisomerase I 24, 25
Topoisomerase II 24, 26, 29, 60
TPA (12-*O*-tetradecanoyl phorbol-13-acetate) 75, 110, 111, 113, 158
Transcriptome 11, 13, 30, 75, 87, 88, 97
Transglutaminases
$TG_c$ 103
$TG_E$ 102–106
$TG_K$ 85, 90, 99–104, 106
Transit amplifier (amplifying) cell(s) 75, 76, 85, 86, 88, 93, 106, 110, 115, 144, 146, 148, 154
Trichohyalin 91, 92, 107
TRSAW fragment, motif 94, 100, 111
TSNARE 192
Tubulin 28, 200
Type II TGF-β receptor 155

## U

Ubiquitination 14, 19, 27, 29, 78, 89, 148, 150, 156
Ubiquitin hydrolase 21, 27
Ubiquitin ligase 16, 18, 21

## V

VDRE (vitamin D-responsive element) 97
VEGF 115
Villi 145, 152
Vinculin 78, 90, 148
Vitamin $D_3$ ($1\alpha$,25$(OH)_2$) 53, 54, 97, 147
Volcano(es) 1–3, 5
VSCCs (voltage-sensitive $Ca^{2+}$ channels) 180, 181, 183, 184, 186–190, 193, 196, 197
VSNARE 192

## W

W-7 naphthalene sulfonamide 56, 59
W-13 naphthalene sulfonamide 56

## Z

Zea mays 233